SUSCEPTIBILITY OF RABBITS TO INFECTION BY THE INHALATION OF VIRULENT PNEUMOCOCCI.

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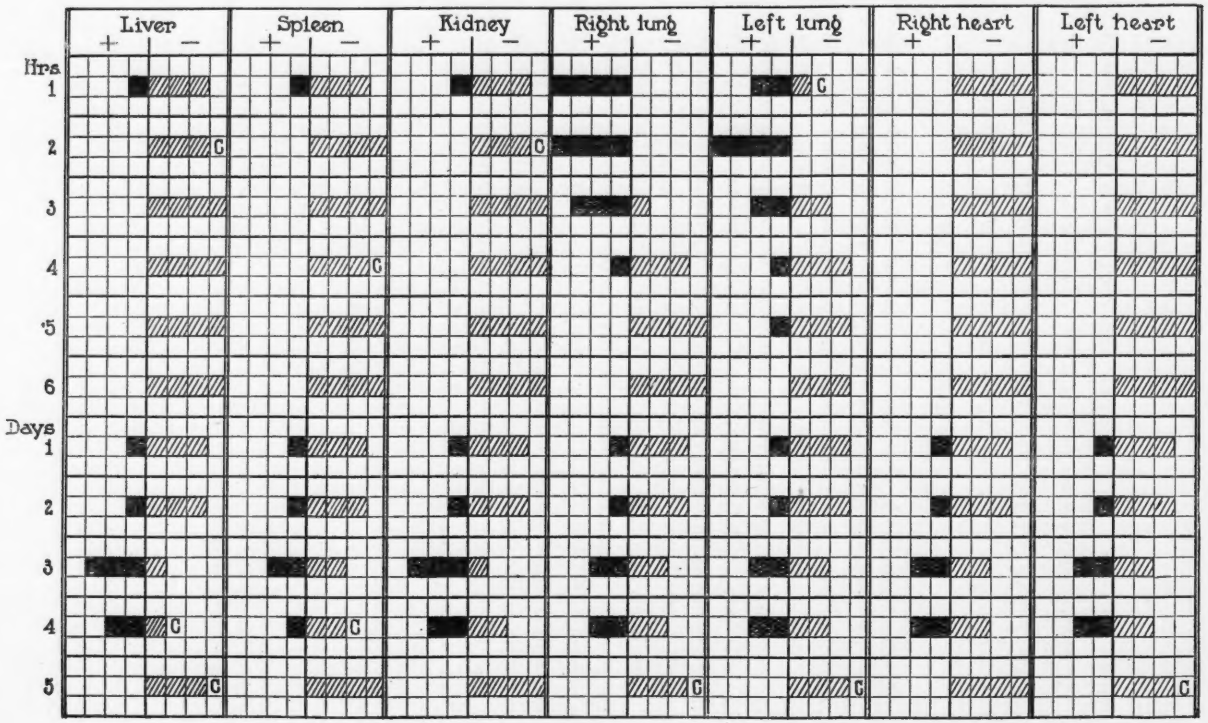
In preceding papers (1, 2) it has been shown that following inhalation, virulent pneumococci generally disappear from the lungs of normal mice within a few hours, that infection rarely occurs, and that in only a few instances does death result from septicemia. It has further been shown that a high degree of active immunity may develop in mice following repeated inhalations of living pneumococci. However, mice are not suitable for tracing serologically the varying degrees of immunity developed by the repeated inhalations. Since rabbits may be easily and repeatedly bled, they were chosen for this work.

In the present paper are reported (1) the distribution of pneumococci in the organs of rabbits following spraying with pneumococci, (2) the occurrence of positive blood cultures, (3) the mortality of the exposed rabbits, and (4) the pathology of the pulmonary lesions in the rabbits which died. The immunity following upon the inhalation of peumococci will be dealt with in a subsequent paper.

Method.

The rabbits were placed in a large spray chamber similar to that already described (2) and exposed to a spray of virulent Type I pneumococci. 150 cc. of an 18 hour broth culture was used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained by bleeding the animal from the ear vein. At varying intervals after the spraying, blood cultures were taken from the ear vein. In the case of the animals which were killed and autopsied, broth cultures were made from both sides of the heart, and small pieces of the periphery of both lungs, and the kidney, liver, and spleen were placed directly in tubes of broth. Heart's blood cultures alone were made from the rabbits which died. All cultures were plated on blood agar for further identification. The animals killed with chloroform were immersed in a solution of lysol and opened with sterile instruments.



TEXT-FIG. 1. Distribution of pneumococci in organs following spraying. The black squares indicate organs in which cultures showed pneumococci to be present, the cross-hatched squares represent organs in which cultures remained sterile.

Distribution of Pneumococci in the Organs.

In Text-fig. 1 is shown the distribution of pneumococci in the organs of 44 sprayed rabbits which were killed at intervals after spraying. It will be seen from the figures for the 24 rabbits killed in from 1 to 6 hours that pneumococci were generally recovered from the periphery of the lungs for the first 3 hours after the spraying but that later they rapidly disappeared. In only 1 rabbit were pneumococci recovered from the liver, spleen, and kidney. The heart's blood cultures from this animal remained sterile. Of the remaining 20 rabbits killed from 1 to 5 days following exposure, 6, or 30 per cent, showed a general pneumococcus septicemia. In 1 rabbit killed on the 3rd day, pneumococci were recovered only from the liver and kidney. This experiment shows how readily normal rabbits may become infected following inhalation of pneumococci. It further indicates that a transient pneumococcus septicemia may be present following spraying. In order to gain further knowledge of this point daily blood cultures were taken from the ear vein of a few rabbits.

Blood Cultures.

From Table I it is seen that following a first exposure positive blood cultures were obtained in 15 out of 44 rabbits. Of the 15 rabbits which developed a septicemia 5, or 33 per cent, continued to have a positive blood culture until death. The other 10 rabbits had merely a transient septicemia and survived. In some instances the septicemia could be detected during the first 24 hours but in others the organisms were first recovered as late as the 6th or 7th day.

Mortality of Rabbits Following Exposure to a Pneumococcus Spray.

Table II shows the number of normal rabbits which succumbed during the course of spraying with virulent pneumococci. From this table it is shown that out of 231 rabbits 80, or 38 per cent, died of a pneumococcus septicemia following their first exposure. Although some rabbits escaped infection at the first exposure, 7, or 6 per cent, died after the second spraying, and an occasional rabbit died after the third and fourth exposures. Of the 112 rabbits which died, the blood of 13, or 11 per cent, was sterile. These rabbits probably succumbed

TABLE I.

Days on Which Positive Blood Cultures Were Obtained Following a Single Exposure to a Pneumococcus Spray.

Rabbit No.	Days..1	2	3	4	5	6	7	8	9	Course
1	c	c	−	−	−	+				Survived
2	−	+	+	−	−					"
3	+	+	+	+						Died 6th day
4	+	−	−	−	−	−				Survived
5	−	+	c	c		+				Died 7th day
6	−	+	+	c	c					" " "
7	−	−	−	c	+		−			Survived
8	+		c			−				"
9	−	−	−	+						Died 6th day
10	−	c	+	+						" 5th "
11	+	c	−	−	−					Survived
12	+	+	−		−	−	−	−	−	"
13	+	−	−		−	−	+	c	−	"
14	−	−	−		−	−	+	−	−	"
15	−	−	−		c	−	+	−	−	"

Blood cultures in 29 rabbits were negative.

A cross indicates a positive culture of pneumococcus, a dash a sterile culture, and "c" a contaminated culture.

TABLE II.

Fate of Rabbits after One to Ten Exposures to Inhalations of Pneumococci.*

No. of times exposed to virulent pneumococci	No. of rabbits exposed	No. dying	Pneumococcus recovered from heart's blood	Pneumococcus not recovered from heart's blood
1	231	86	80	6
2	111	9	8	1
3	98	11	9	2
4	71	4	2	2
5	50	1	−	1
6	39	1	−	1
7	34	−	−	−
8	31	−	−	−
9	31	−	−	−
10	27	−	−	−

* The discrepancy between the total numbers and the sum of the numbers in the individual groups is due to the fact that certain of the animals that survived were used for intraperitoneal tests or for other experiments.

to suffocation from being enclosed in the spray boxes too long. This technical error was corrected in later experiments. The table also brings out the fact that the greatest number of deaths occurred after the first exposure. After each successive spraying the mortality tended to lessen until after the fourth exposure no animals succumbed. In other words, those rabbits which were most susceptible to pneumococcus died early. As will be shown in a subsequent paper, the surviving rabbits were those which not only had the greatest natural resistance but also had acquired a certain degree of immunity as a result of repeated exposures to pneumococcus.

The interval elapsing between the time of spraying and death from pneumococcus septicemia is shown in Table III. From this it is seen that the greatest number of animals died within the first 7 days fol-

TABLE III.

Number of Days Elapsing between Spraying and Death of Rabbits from Pneumococcus Septicemia.

	Days..1	2	3	4	5	6	7	8	9	10
Spray I.....	3	12	16	8	14	12	8	5	1	1
" II.....	—	2	—	3	1	—	—	—	1	1
" III.....	2	—	2	—	—	1	2	2	—	—
" IV.....	—	—	1	—	—	1	—	—	—	—

lowing the initial exposure, although an occasional rabbit survived longer. The time of death of the rabbits which had survived the first exposure is much more diverse, and these rabbits have a tendency to die after a longer interval.

Pathology.

In no instance was there any gross evidence of pneumonia, although serous and serofibrinous pleurisy and serofibrinous pericarditis were common. A histological examination was made of the lungs of 77 rabbits which died of pneumococcus septicemia following spraying. The lungs appeared normal in 20. Congestion and pleurisy were noted in 7 instances, edema in 25, congestion in 38, and hemorrhagic extravasation into the alveoli in 19. In only 1 instance was there any histolog-

ical evidence of an attempt at localization of the infection. The lungs of this rabbit which died on the 4th day following the first exposure showed an interstitial inflammation (3).

DISCUSSION.

From the foregoing experiments it appears that rabbits are even more susceptible than mice to infection with pneumococci following inhalation. For a period of 3 hours after spraying the organisms may be demonstrated at the periphery of the lungs. It is unfortunate that their subsequent course deeper into the lungs cannot be followed. But the great number of other organisms which normally inhabit the bronchi of rabbits, as Jones (4) has shown, renders cultural studies of the lungs difficult. Histological detection of pneumococci in the tissues is also unreliable when the organisms are present only in small numbers.

In all probability the majority of the organisms are destroyed either when they first localize or deeper in the lung tissue by the endothelial leucocytes or by polymorphonuclear leucocytes. In any case a few organisms occasionally filter through into the blood stream. Gaskell (5) believes that invasion of the blood stream by pneumococci probably always takes place in the early stages of an air-borne infection of the lung. The actual invasion of the blood stream must be much more common than is supposed. Not only are there probably few organisms free at any one time in the circulating blood but these few bacteria may even be within leucocytes and not in reality multiplying in the blood.

The occasional organisms which reach the blood are probably rapidly filtered out of the blood stream by the organs and locally destroyed. It is evident, however, that following the apparent disappearance of the pneumococci from the periphery of the lungs, they again appear, this time in the blood, and as a result may be recovered from all the organs. The point of this reinvasion is as yet uncertain.

Following the initial spraying with pneumococci the majority of susceptible rabbits die of an overwhelming septicemia without any attempt on the part of the body to localize the infection.

CONCLUSIONS.

1. Rabbits are very susceptible to infection by inhalation of Type I pneumococci.

2. When rabbits are exposed to a pneumococcus spray the bacteria readily penetrate into the lower respiratory tract. The pneumococci which reach the periphery of the lungs as a result of this procedure usually disappear within a few hours but a generalized and fatal septicemia frequently later appears. Pneumococci may then be recovered from the periphery of the kidney, liver, and spleen. In the animals which die pleurisy and pericarditis are common but pneumonia does not occur.

3. Rabbits may recover from pneumococcus septicemia.

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STUDIES IN EXPERIMENTAL SYPHILIS.

VI. On Variations in the Response of Treated Rabbits to Reinoculation; and on Cryptogenetic Reinfection with Syphilis.*

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The decision in any given case as to whether or not a syphilitic animal, treated and then reinoculated, has acquired a second attack of syphilis, will, of necessity, depend upon the criteria of a successful reinfection. In the older experimental work attention was directed mainly, if not entirely, to the occurrence of a local lesion (chancre) at the site of reinoculation. If no such lesion developed in the reinoculated animal it was assumed that a second infection had not been established. It is at once apparent that this assumption is justified only if syphilitic infection is always accompanied by the occurrence of a primary lesion at the portal of entry. That syphilis may occur in human beings without the development of a chancre is well known (1). The same has been shown to hold true for monkeys (2) and for rabbits (3–5). In view of these now well established facts it is clear that, in determining in the reinoculated animal whether or not a second infection has been established, one must take into consideration the possibility that second infections may occur without the appearance of any local lesion at the portal of entry. Such second infections might be characterised by dissemination of the treponemata throughout the body and their lodgement in distant organs, with or without the production of lesions at the site of such lodgement. Neisser (2) admitted the possibility of such a result from reinoculation in his discussion of superinfection,

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and Brown and Pearce (6) called attention to this point several years ago. As they very aptly expressed it, one must be able "to see beyond the reaction at the site of reinoculation." More recently the same question has been raised by other workers in this field (7–9).

During the past few years in the course of a series of experiments, some of which have been reported in other communications, we have had occasion to carry out a number of reinoculations in treated syphilitic rabbits. Keeping in mind the principle elaborated above, we directed our attention, in judging of the results of reinoculation, not alone to (*a*) the occurrence of a lesion at the site of reinoculation, but also to the possibility of (*b*) the establishment of infection without the production of a local lesion, to (*c*) the occurrence of dissemination of the virus under these conditions, and to (*d*) the production of a positive Wassermann reaction. The purpose of this communication is to bring together the results of reinoculation of treated rabbits studied from these points of view. The evidence afforded by this study indicates that it is possible successfully to reinoculate a treated rabbit without the production of any clinically detectible lesion at the site of reinoculation; furthermore, that the time at which treatment is begun and the method of reinoculation are of importance in bringing about this type of response to a second infection.

In this communication we shall deal only with the results in treated rabbits, reinoculated with homologous strains of *Treponema pallidum*. It is obvious that in untreated rabbits reinoculated with the same strains it would be a most difficult feat to demonstrate a successful reinoculation if such occurred without a lesion being produced at the second portal of entry.

In the first part of our report we shall discuss the results obtained when both first and subsequent inoculations were intratesticular, and in the second part we shall deal with the results obtained when the first inoculation was intratesticular and the second was made by inoculating a granulating wound produced on the back of the animal. The Nichols strain was used in all the experiments.

I. All Inoculations Intratesticular.

Experimental.

First Inoculation.—In each animal only one testis was inoculated with virus emulsion.

Treatment.—Arsphenamine was administered intravenously at weekly intervals for a total of six doses. The individual dose was 10 mg. per kilo in each instance. Treatment was begun at varying intervals after inoculation, ranging from 41 to 291 days. Lymph node transfer was performed in all but one of the animals *after* treatment but *before* reinoculation.

Reinoculation.—Reinoculations were in some instances performed only once and in others twice, and always in the testis opposite to that originally inoculated. They were carried out 53 to 69 days after treatment was concluded. Lesions developing at the site of reinoculation were, of course, examined for the presence of treponemata. If no lesion developed the testis was excised, an emulsion of the organ was made with normal saline solution and examined for treponemata by the dark-field method. In four instances where this emulsion was negative it was inoculated intratesticularly into normal rabbits. In every instance the reinoculated testis was studied by one or both of these methods.

Dissemination of Virus.—The possibility of occurrence of dissemination of the virus was ascertained by clinical observation and by transfer of a popliteal lymph node to the testes of normal rabbits. This was carried out from 90 to 101 days after reinoculation and it was not omitted in any animal reported upon in this paper. The period of observation following reinoculation was at least 90 days and in many instances longer. As judged by the behavior of the controls this period affords ample opportunity for the development of syphilitic lesions at the site of reinoculation.

Wassermann Reaction.—The Wassermann reaction was performed on the blood serum of many of the rabbits at frequent intervals before and after reinoculation, according to the technique outlined in the preceding paper of this series. The reaction was negative in every instance prior to reinoculation.

Results.

We are able to report upon a total of twenty-three treated syphilitic rabbits reinoculated and studied as described above.

If it be assumed that reinfection may take place without the production of a local lesion at the site of reinoculation, but may be manifested by local infection without clinical phenomena, or by dissemination of the virus, then it is apparent that there are six theoretically possible responses to reinoculation, ranging from the production of a second infection which is entirely similar to the first,

to no infection at all. These six theoretical possibilities may be stated as follows:

Response A.—The production of a local lesion at the site of reinoculation accompanied by dissemination of the virus, in other words, a reaction which is identical with that of normal rabbits to syphilitic infection.

Response B.—The production of a local lesion at the site of reinoculation without evidence of dissemination of the virus.

Response C.—The persistence of virus at the site of reinoculation and dissemination of the virus but without the development of any local lesion.

Response D.—Dissemination of the virus without the production of a local lesion and without the persistence of virus at the site of reinoculation.

Response E.—Persistence of the virus at the site of reinoculation without dissemination and without the development of a local lesion.

Response F.—Absence of a local lesion and absence of virus at the reinoculation site together with no dissemination of the virus; in other words, a negative result.

It is, of course, questionable how far one may go in distinguishing Response D from Response C, since it is always conceivable that the virus may have been present at the site of reinoculation but have been overlooked due to insufficient examination. Negative results are notoriously misleading, but the theoretical possibilities exist, and it is of interest to see how closely the observed facts fit them, when reasonably diligent attempts are made to find the organisms at the site of reinoculation.

Of the six possible responses to reinoculation, examples of five were encountered in one or more rabbits in the group of twenty-three reported upon. The distribution of these rabbits among the five responses, or categories, as we shall call them, is shown in Table I, together with the duration of the infection as well as the duration of the initial orchitis at the time treatment was begun.

Consideration of Table I shows that in six of the entire series of twenty-three animals a characteristic local lesion developed at the site of reinoculation, followed by evidence of dissemination of the virus (Category A). The duration of the primary orchitis and the

TABLE I.

Incidence of Various Types of Response to Reinoculation.

Category	Lesion at site of reinoculation	Virus at reinoculation site without lesion	Dissemination of virus (node transfer)	Rabbit No.	Duration of orchitis at time of treatment	Duration of infection at time of treatment	Wassermann reaction after reinoculation
					days	*days*	
A	+	0	+	1	17	49	Not done
				2	22	48	" "
				3	16	48	" "
				4	15	48	" "
				5	16	41	++++
				6	26	41	++++
				Total 6	Average 18.7	Average 45.8	
B	+	0	0	None	—	—	
C	0	+	+	7	23	41	++++
				8	32	68	++++
				Total 2	Average 27.5	Average 54.5	
D	0	0	+	9	16	41	++
				10	16	61	++
				11	26	41	++++
				12	26	41	++++
				13	19	41	++++
				14	38	68	++++
				Total 6	Average 23.5	Average 48.8	
E	0	+	0	15	167	193	0
F	0	0	0	16	179	209	Not done
				17	196	209	" "
				18	272	291	" "
				19	27	56	0
				20	182	193	0
				21	174	193	0
				22	174	193	0
				23	171	193	0
				Total 8	Average 172	Average 192	

duration of the infection at the time treatment was instituted averaged 18.7 and 45.8 days respectively. The Wassermann reaction of the two animals of this group in which it was performed became positive after reinoculation. None of these animals exhibited generalised lesions following reinoculation.

There were no instances in which a local lesion was produced at the site of reinoculation without dissemination of the virus (Category B). In our experience whenever a local lesion has been produced with the Nichols strain at the site of reinoculation, it is invariably accompanied by dissemination of treponemata and involvement of distant lymph nodes.

In two animals (Category C) no local lesion was produced but careful search revealed the presence of treponemata at the site of reinoculation and there was definite evidence of dissemination of the virus. The average duration of the primary orchitis and of the infection at the time treatment was instituted in these animals was 27.5 and 54.5 days respectively. Both animals developed positive blood Wassermann reactions after reinoculation.

In six animals (Category D) no local lesion developed following reinoculation nor was it possible by careful search to demonstrate the presence of treponemata *in situ*, nevertheless, there was evidence of dissemination of the virus in all of them and they all showed positive blood Wassermann reactions following reinoculation. The average duration of the primary orchitis and of the infection at the time treatment was begun was 23.5 and 48.8 days respectively.

We encountered one rabbit, No. 15 (Category E), in which no local lesion developed at the site of reinoculation and in which there was no evidence of dissemination of the virus as judged by lymph node transfer or the occurrence of generalised lesions. Careful search of the testis (left) in which the reinoculation was made showed no treponemata by dark-field examination, but the emulsion of testicular material was inoculated into two normal rabbits and both developed syphilis. It should be noted that this animal was originally inoculated in the right testis and a characteristic syphilitic inflammation of this organ developed, but up to the time treatment was instituted, namely, 193 days after the original inoculation and 167 days after the primary orchitis was manifest, no macroscopic lesions had appeared

in the opposite testis (left), which was the one reinoculated. Furthermore, this animal never developed a positive Wassermann reaction after reinoculation. The evidence is very suggestive that the virus recovered by animal inoculation from the reinoculation site was that introduced at the time of reinoculation. It would appear that the animal had been rendered highly, although not completely refractory to a second infection.

There were eight rabbits in which no local lesion developed at the site of reinoculation and in which the virus could not be demonstrated at that site (Category F). Furthermore, the virus could not be recovered from the lymph nodes of any of these animals following reinoculation and in all of them the Wassermann reaction remained persistently negative. Of the eight animals in this group, there were four in which the reinoculated testis was emulsified and, in addition to being studied by dark-field examination, was inoculated intratesticularly into each of two normal rabbits. In every instance this inoculation proved to be negative. In the remaining four animals the examination of the reinoculated testis was confined to dark-field examination of an emulsion of the organ. In this group, then, no evidence could be adduced to show that any of the animals had been successfully reinoculated. The average duration of the infection in this group at the time treatment was begun was 192 days. All but one of the animals falling in this group were treated late in the course of the disease. The single exception, No. 19, received its first treatment on the 56th day of the infection.

Comment.

The foregoing results indicate that when rabbits with syphilitic orchitis are treated with arsphenamine comparatively early in the course of the disease, that is to say before the 69th day, and are subsequently reinoculated intratesticularly in the opposite testis with the homologous strain of treponemata, the response to the second inoculation will vary within stated limits. Some animals may show a response similar to that of normal animals and are, therefore, to be regarded as non-immune. Other animals may show no local lesion at the reinoculation site but, nevertheless, will present evidence of having been successfully reinoculated as determined by the persistence

of the virus at the site of reinoculation and its dissemination to distant lymph nodes. Still others may show no virus at the reinoculation site but give evidence of its dissemination nevertheless. In general, it would appear that reinfection of treated syphilitic rabbits may take place without any local lesion, following intratesticular reinoculation, and that this type of response to a second inoculation is apt to occur when treatment is begun from 41 to 68 days after the first infection. It would appear to be almost constantly accompanied by the development of a positive Wassermann reaction in the blood. Animals responding in this manner to a second infection may conceivably be regarded as in part refractory. Occasionally, as exemplified by Rabbit 19, an animal may become apparently entirely refractory to a second infection early in the course of the infection and remain so even if treatment is begun before the 69th day of the infection.

The behavior of Rabbit 15 is of interest in that it indicates a high degree of resistance to a second infection, which, however, falls just short of being complete. This animal was treated late in the course of the disease (193 days) and when reinoculated gave no indication of dissemination of the virus, but the presence of the latter at the site of reinoculation could be demonstrated by animal inoculation. The animal, moreover, failed to exhibit a positive Wassermann reaction following reinoculation.

Where the treatment was postponed for 6 months or more there was, except in the one instance noted above, no evidence that a successful reinfection had taken place. This observation is in accord with those of Kolle (10) and Frei (11) and would indicate that the refractory state in such animals is probably complete.

The data presented in Table I would indicate that in general the type of response (A) to a second inoculation characterised by the development of the essential disease picture (local lesion and dissemination of the virus) is most apt to occur when the primary orchitis is interrupted relatively early (average 18.7 days) by treatment. If treatment is withheld until the orchitis is slightly farther advanced (average 24.5 days) the response tends to become that characterised by dissemination of the infection without the production of a local lesion (D and E). If treatment is withheld until a much later date

(6 months or more), when the animal has had opportunity to heal the initial lesion by virtue of its own defensive mechanism, no infection occurs following intratesticular inoculation, or at most a local infection without local lesion and without dissemination of the virus. These facts point to a gradually developing resistance, the first manifestation of which is the ability of the animal to suppress the local reaction.

It may be objected that the recovery of the virus from distant lymph nodes of treated reinoculated animals in which no local lesion is produced at the reinoculation site, is not conclusive evidence of the success of reinoculation, since it is conceivable that the virus recovered from such nodes may be that of the first infection. This objection may be met with the statement that this phase of the experiment was controlled in all but one of the animals by removal of a node *after* treatment but *before* reinoculation, and transfer of the latter to normal animals. All such transfers proved to be negative. Furthermore, study of other animals similarly treated but *not* reinoculated showed that treatment was uniformly effectual in rendering the nodes non-infectious. Even if nodes were removed from such rabbits as late as 201 days after the last dose of arsphenamine they always failed to convey the infection to normal animals. Similar results were obtained by Nichols and Walker (12) with even fewer doses of arsphenamine than we have used. In addition, the occurrence of a positive blood Wassermann reaction following reinoculation, when previously it had become negative under treatment, offers additional support for the view that such animals were in truth reinoculated successfully and that the virus obtained from the lymph nodes did not originate from the first reinfection but represented that introduced at the time of the second inoculation.

II. Reinoculations on Wounds.

In a previous communication (13) we have called attention to the fact that a granulating wound (11 to 16 days old) on the back of a rabbit offers a particularly favorable site for the development of a chancre after inoculation with the Nichols strain of *T. pallidum*. When a testicular emulsion of the virus is allowed to drop on the exposed granulating surface of such a wound, infection readily takes

place and after the wound heals a characteristic chancre develops, which can even be made to conform to a predetermined pattern. It seemed of interest to determine how a rabbit, first infected by intratesticular inoculation and treated at a time when it could be presumed to be refractory to a second intratesticular inoculation, would react to a second infection in which the virus was deposited upon a granulating wound on the back, in the manner previously described.

It is at once apparent that such an experiment might throw light upon the question of the extent to which the refractory state, which develops in syphilitic rabbits in the course of time, is a property of the animal body as a whole, that is to say, a general resistance, as against a local resistance only. There is in the literature some evidence to show that not all of the tissues of the rabbit share equally in the resistance toward a second infection which syphilitic rabbits unquestionably acquire.

Tomasczewski (14) found that if rabbits were inoculated in the cornea with syphilitic virus and a syphilitic keratitis developed, such rabbits, in most instances, could be successfully infected a second time by subscrotal inoculation as late as 128 days after the appearance of the keratitis. He also found that if the first infection were produced by subscrotal inoculation it was possible to produce a syphilitic keratitis in 76 per cent of his animals by inoculating the cornea from 39 to 105 days after the first inoculation. Apparently he reinoculated with an homologous strain of treponemata. Uhlenhuth and Mulzer (15) also succeeded, in three of four experiments, in obtaining second infections by intratesticular inoculation when the first infection was produced by corneal inoculation. In contradistinction to Tomasczewski, however, they were unable to obtain, in eleven of thirteen experiments, a second successful infection by the corneal route when the animals had been previously inoculated intratesticularly and a characteristic syphilitic orchitis had been produced. In two of their animals, however, they did succeed in producing a keratitis following testicular inoculation. The interval elapsing between first and second inoculations in their experiments ranged from 40 to 170 days. The two successful second infections were obtained when the second inoculation was carried out 40 and 61 days, respectively, after the first. These investigators were apparently using homologous strains for first and second inoculations.

Zinsser (16), largely on the basis of these experiments together with some of his own, has formulated the principle that the refractory state which develops in syphilitic rabbits is in reality a local phenomenon dependent upon previous infection at the site of reinoculation. Thus, his conception of syphilitic immunity is restricted even more than that of Kraus and Volk (17) who have suggested that it is a tissue immunity restricted to certain tissue groups and not shared to the same extent by all tissues.

Experimental.

Sixteen animals were inoculated intratesticularly with the Nichols strain of *T. pallidum* and the infection was permitted to run its course for a period of more than 5 months. All the animals were then treated with arsphenamine intravenously in exactly the same manner as in the preceding experiment. Treatment was begun 159 to 201 days after inoculation, the average being 169 days.

TABLE II.

Reinoculation of Treated Rabbits.

Rabbit No.	Wassermann reaction before reinoculation	Result of reinoculation			
		Lesion at site of reinoculation	Generalised lesions	Wassermann reaction	Node transfer
24	Negative	None	None	Negative	Negative
25	"	"	"	"	Positive
26	"	"	"	"	Negative
27	"	"	"	"	Positive
28	"	"	"	"	Negative
29	"	"	"	"	"
30	"	"	"	Positive	"
31	"	Abscess	"	"	Positive
32	"	Chancre	"	"	"
33	"	None	"	Negative	Negative
34	"	Abscess	"	Positive	Positive
35	"	None	"	Negative	"
36	"	"	"	"	"
37	"	"	"	"	Negative
38	"	"	"	"	Positive
39	"	"	"	Positive	Negative
Virus controls					
40	"	Chancre	"	"	Not done
41	"	"	"	"	" "
42	"	"	"	"	Positive
43	"	"	"	"	Not done
44	"	"	"	"	Positive

89 days after the last dose of arsphenamine was administered the animals were reinoculated on the back. This was accomplished by first excising an elliptical area of skin, under ether anesthesia, exposing the subcutaneous tissue. The wounds were not dressed in any way and 14 days after operation, when granulation tissue was well established, the crusts were removed with as little trauma as possible and 2 drops of a testicular emulsion, rich in actively motile treponemata, were allowed to drop on the exposed granulating surface. The same

strain (Nichols) of treponemata was used for reinoculation. The virulence of the inoculum was controlled by inoculating five normal rabbits with wounds of the same age and prepared in the same way. Reinoculation was performed 248 to 290 days after the first inoculation. Wassermann reactions were performed at frequent intervals both before and after reinoculation. In addition a single popliteal node was removed from each test animal and from two of the virus controls 60 to 63 days after reinoculation; it was emulsified in normal saline solution and the entire emulsion inoculated into the testes of each of two normal rabbits. The test animals were observed for a period of 195 days following reinoculation before the experiment was terminated.

Results.

The results are shown in Table II.

In all of the animals the wounds healed at about the rate expected in uninoculated rabbits. In two of the test animals abscesses developed and these were incised and drained. No treponemata could be demonstrated in the exudate from these infected areas. In one animal (No. 32) a characteristic chancre developed after an incubation period of about 25 days. Treponemata could be demonstrated with ease in the serum obtained from this lesion. It is of interest that this chancre began to regress spontaneously 47 days after its appearance, long before there was any sign of spontaneous regression in the lesions which developed in the controls. There were never any signs of generalised syphilis in this rabbit but the Wassermann reaction became positive after reinoculation and the lymph node transfer was also positive. In no other rabbit did a syphilitic lesion appear at the site of reinoculation. It is true that several animals exhibited transient induration of the scar at about the time of complete healing, but serum from these areas did not show treponemata by dark-field examination and no lesions developed subsequently. In five rabbits, including the one in which the chancre developed, the Wassermann reaction became positive. Lymph node transfer was positive in three of these five animals. Altogether, in eight of the sixteen test animals lymph node transfer was positive and in only one of these did a syphilitic lesion develop at the site of inoculation. In none of the sixteen test animals did any generalised syphilitic lesions make their appearance.

In the five control animals a typical chancre appeared in each

instance. These went through the ordinary course of development such as we have described previously, and finally healed spontaneously. In all five the Wassermann reaction became positive and lymph node transfer in the two instances in which it was done was positive. No generalised lesions were noted in the controls during the period of observation.

Comment.

It is clear that in but one of these sixteen treated and reinoculated rabbits did there develop a characteristic chancre following reinoculation upon a granulating wound. The remaining fifteen failed to show anything at the site of reinoculation which might be considered as syphilitic, and yet, in seven of these fifteen, lymph node transfer was positive, indicating that the animals were syphilitic at the time transfer was performed. For reasons outlined above we are inclined to the view that the virus obtained from these lymph nodes was that introduced at the time of reinoculation, and we interpret the experiment as indicating that at least half of the test animals had been successfully reinfected by inoculating a granulating wound on the back although in only one did a syphilitic lesion develop at the portal of entry.

The high incidence of successful reinoculations obtained in late treated syphilitic rabbits when the virus was deposited upon a granulating wound on the back (50 per cent), as contrasted with the comparatively low incidence of successful reinoculations obtained when the virus was injected intratesticularly, calls for explanation. One possibility that has to be taken into consideration involves the contention of Neisser that acquired immunity to syphilis is dependent upon the persistence of active foci of syphilitic infection somewhere in the body of the host, and furthermore that the response of a treated animal to reinoculation can be taken as an index of cure. In previous publications we have discussed this theory and need not therefore consider its validity at this point. Assuming it to be true, the results outlined in the second experiment are susceptible of but one interpretation, namely, that although treatment was begun late in the disease half of the animals were cured while half were not. On the same basis it would have to be concluded that the same mode of

treatment carried out in another group of syphilitic rabbits at a comparable period in the course of the disease (Category F, Table I) had failed to bring about cure in any, since they were all refractory to a second inoculation. This explanation does not seem logical and has the disadvantage in that it gives no clue as to why identical treatment should fail completely in one experiment and be 50 per cent effective in another, other things being equal. Nevertheless, if it is correct, then the experiment necessarily indicates that treatment late in the course of the infection in rabbits will apparently cure at least half of the animals.

A second and perhaps more plausible explanation which is suggested is that the resistant state which develops in syphilitic rabbits as a result of their infection is not absolute but is capable of being broken down in part by resort to a method of inoculation which appears to be particularly favorable to the inciting agent. It is possible that this breaking down of the animal's resistance is accomplished through failure of the granulation tissue of these healing wounds to share in the resistant state to the extent as does the testis for example, whether or not the latter organ has previously been the seat of a syphilitic inflammatory process. Nevertheless it would seem that the newly formed granulation tissue does share to some extent in the immune process since half of the reinoculated animals were, so far as we could judge, not reinfected, and since in only one of the remaining half, which apparently were successfully reinfected, did a characteristic chancre develop in the wound.

It is entirely possible that the tissues in these granulating wounds in reality did possess a high degree of resistance to a second infection but that the trauma incidental to removal of the crust of the wound preparatory to inoculation resulted in the opening up of lymph channels and thus facilitated migration of the treponemata and invasion of the blood stream before any local defensive process could effect a complete destruction of all the treponemata. If this is the correct explanation of the apparently successful reinfection of half of the animals, it would speak against a humoral immune mechanism in syphilitic rabbits, at least of great magnitude.

It is necessary to consider also the possibility that there is no real resistance to reinfection in these granulating wounds in late treated

syphilitic rabbits, that is to say no active local defensive mechanism. It may be that all that is being observed is an instance of acquired inability of tissues to react in the customary manner to syphilitic virus, that is, an indifference or state of anergy in the sense that Neisser used the term. If such is the correct view then we must consider the possibility of a humoral factor in acquired resistance to syphilis since at least half of the test animals were apparently refractory to a second infection. Regardless of whether one considers this inability to produce a characteristic chancre by inoculating granulating wounds of late treated syphilitic rabbits as evidence of tissue indifference or anergy, or as evidence of the existence of a local active defensive mechanism more or less complete, it must be admitted that the acquired property of the granulation tissue not to react is not dependent upon a preexisting local syphilitic infection. By no stretch of the imagination can one conceive of such granulation tissue having been previously infected with syphilis since the wounds were made after the animals had been thoroughly treated. The more or less refractory state of the granulating wound in the immune animal may be dependent upon either a humoral defensive mechanism, or a mechanism limited to cells, or a combination of both factors. Whichever factor is operative, the experiment shows clearly that the resistant state which develops in rabbits during the course of syphilitic infection is in part at least conferred upon newly formed granulation tissue (with all that that term implies) and suggests that the immune mechanism, if cellular, is capable of being inherited by, or imparted to, newly formed cells. The experiment suggests also that a method is at hand for successfully reinfecting syphilitic rabbits which from past experience one would be justified in assuming to be refractory to an intratesticular inoculation.

Whatever is the correct explanation of the facts that have been observed, it seems clear that in considering the results of reinoculation of treated syphilitic rabbits the experiments cited above would indicate that it is essential to bear in mind that the time at which treatment is begun and the mode of reinoculation are important factors in determining the character of the response to a second infection. Furthermore the evidence is strong that under the conditions of the experiments a second syphilitic infection with the homologous strain

of treponemata may be produced in a large proportion of treated rabbits without the development of any syphilitic lesion at the site of reinoculation.

The results of the experiment recorded in Table II, when contrasted with those outlined in Table I, seem to throw additional light upon the question of the advisability of using the reinoculation method as a criterion of the cure of syphilis. Our previous experiments, in conformity with those of Kolle and of Frei, have shown that when rabbits are infected by the intratesticular route and are treated with arsphenamine late in the course of the infection (181 to 291 days) they are almost uniformly (over 90 per cent) refractory to a second infection introduced in the testis. According to the reinoculation test these animals were not cured. On the other hand, in a group of sixteen animals originally inoculated in the testis and treated 159 to 201 days after infection with the same drug and the same dosage, successful reinoculations (with the same criteria) were apparently accomplished in eight, or half of the animals, when the second inoculation was made upon a granulating wound on the back of the animal. Using the reinoculation test as a criterion of cure one would have to assume that half of these animals had been cured and the other half had not. If comparable groups of rabbits treated in exactly the same manner can give such different results when reinoculated in different ways (90 per cent or more against 50 per cent), how is it possible in such experiments to draw any deductions, from the result of the reinoculation, as to the persistence or absence of the first infection in the body of the host, that is to say, cure or failure to cure? Who is to say which method of reinoculation is the one by which to judge? One can scarcely escape the conclusion that, in view of the variable results given by different methods of reinoculation, such a procedure cannot be accepted as a valid method for determining the possibility of cure of syphilitic infection.

It is perhaps not amiss to point out that the experiments outlined above may have clinical significance. If it is true that under certain conditions a treated syphilitic rabbit can be successfully reinfected without the occurrence of a local lesion at the site of reinoculation, but, nevertheless, with the development of a positive Wassermann reaction in the blood and with dissemination of the treponemata,

then one may well raise the question whether such a phenomenon ever occurs in human beings. Every syphilologist is familiar with the patient who comes under treatment relatively early, who is systematically treated, whose Wassermann reaction becomes negative, and who subsequently, months or years after the treatment has ceased, suddenly shows a completely positive Wassermann reaction in the blood without any signs of syphilis anywhere. Such cases have in the past been regarded usually as instances of a serological relapse, and, by inference, of a failure to cure. In the light of our experiments it may well be that such cases in some instances represent examples of a second infection without signs of syphilis, rather than instances of Wassermann reaction recurrence, and that this conceivable modification of the usual response to syphilitic infection, which they exhibit, is the result of the influence of the first infection. It is universally admitted that second attacks of syphilis with all the characteristic phenomena of a first infection do occur, although rarely. It is not such a far cry to imagine that second infections may take place in treated individuals, with no more evidence of their existence than the development of a positive Wassermann reaction. The importance of deciding whether such cases are really instances of a new infection, rather than examples of a relapse of the old, must be manifest so long as reinfection in humans is regarded as adequate evidence of cure. The clinical differentiation of a case of Wassermann reaction relapse from that of a reinfection without manifest signs but attended by the development of a positive Wassermann reaction, assuming that it occurs in man, would probably be an almost insurmountable task.

SUMMARY.

Syphilitic rabbits inoculated intratesticularly and treated with arsphenamine before the 69th day of the disease, when reinoculated with the same strain of treponemata and in a manner identical with that of the first inoculation, are capable of responding to the infection in at least five different ways. In addition to exhibiting a local lesion at the site of reinoculation, accompanied by dissemination of the virus, they may show no local lesion at all but present evidence of dissemination of the virus together with the development of a positive Wassermann reaction. In some instances the virus may

be recovered from the reinoculation site although no local lesion is produced there and no dissemination of the virus can be shown to take place. An occasional animal treated before the 69th day of the disease remains completely refractory to a second infection.

When treatment is postponed to 6 months or more after the original inoculation, reinfection carried out by intratesticular injection is almost always impossible and such animals appear to be entirely refractory. However, if rabbits treated late are reinoculated with the homologous strain by depositing the virus upon a granulating wound on the back, successful reinfections can be accomplished in at least 50 per cent of the test animals. The resistance which develops in rabbits in the course of syphilitic infection is, then, not absolute but relative.

It is pointed out that these results cast discredit upon the validity of the reinoculation method as a test of cure in syphilitic infection. It is also suggested upon the basis of these experiments, that the subsequent occurrence of a positive Wassermann reaction in patients with early syphilis in whom the Wassermann reaction has become negative under treatment may not always represent a relapse in the the disease but possibly in some instances a new infection without clinical signs.

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STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VI. On the Virulence of the Overgrowth in the Lysed Cultures of Bacillus pestis caviæ (M. T. II).

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When susceptible bacteria are grown on broth in the presence of a suitable lytic agent, the initial turbidity of the culture gradually disappears because of the lysis of the bacteria. In exceptional cases the lysis may be complete and permanent, resulting in sterilization of the culture. But more often a few bacteria fail to undergo lysis and multiply in spite of the presence of lytic agent in the medium, thus giving rise to more or less profuse overgrowth of resistants, following a temporary clearing of the culture (1).

In the case of *B. coli*, Bordet and Ciuca (2) found that the overgrowth differs from the original culture, in addition to its resistance to lysis, by its active motility; and Gratia (3) found further that the overgrowth is more highly virulent. The heightened virulence of bacteria resistant to lysis was observed also by other investigators and has been suggested as the source of the failures of bacteriophage to influence favorably the course of infection in certain instances (4–7).

In view of the fact that the virulence of a strain M. T. II of *B. pestis caviæ* was carefully established during several years of study of experimental mouse typhoid at The Rockefeller Institute (8–10), we thought that a lysis-resistant overgrowth of this culture would offer exceptional material for comparison of its virulence with that of the original culture.

Isolation of Resistants.

In order to obtain pure strains of resistant bacteria, the stock culture M. T. II was grown in broth for 24 hours, in the presence of each of the two lytic filtrates

active against this organism. These lytic filtrates were selected from several isolated by us at different times from the stools of mice recovering from experimental mouse typhoid, because they were typical of the variations in the character of lytic activity observed in these filtrates.[1]

The overgrowth which was present in the cultures after 24 hours of incubation at 37°C. was streaked out on agar plates. Single colonies appearing on the plates were repeatedly transferred to fresh agar (each time from a single colony) in order to eliminate all traces of lytic principle. After seven successive transfers, 24 hours apart, each of the cultures was returned to broth. They proved to be free from lytic principle when filtered and tested against the original susceptible stock culture of M. T. II. Broth cultures themselves were tested at the same time for their susceptibility to lysis by each of the lytic filtrates in turn. As the results recorded in Protocol I demonstrate, each culture obtained from the overgrowth was resistant to lysis by the lytic agent used in its production, but only one of the cultures was resistant also to the activity of the second lytic agent.

Protocol I.

Identification of Resistants Isolated from Lysed M. T. II Culture.

Lytic agents used to obtain resistants	Character of growth in broth	Agglutinability with stock M. T. II serum 1:2000	Resistance or susceptibility to lysis	
			Resistant W–Little R_7*	Resistant W–178 R_7*
W–Little.....	Agglutinated	Positive	Resistant	Resistant
W–178.....	Diffuse	Positive	Susceptible	Resistant

* The legend R_7 added to the name of each culture indicates that it represents a resistant (R) variant of the seventh (7) passage on agar.

The identity of these cultures with the parent culture of M. T. II was checked by the agglutination test, in which a stock anti-M. T. II serum diluted 1:2000 was used as agglutinin.

It was observed that in some cases the overgrowth appeared diffuse; in others it was spontaneously agglutinated. Since this difference in the appearance of overgrowth has been stressed in the literature (6, 7) we have carried out all the experiments with Strains W-178 and

[1] We are greatly indebted to Dr. Ida Pritchett for placing at our disposal the greater part of the animals used for isolation of lytic agents. In passing, it should be recorded that the lytic filtrates yielded by the examination of over 300 mice were, for the most part, active only against *B. dysenteriæ*. Next in frequency were those active against *B. enteritidis* Gaertner, and least frequently those active against the infecting organism M. T. II.

W-Little as representing this difference in growth. In order to avoid spontaneous return of susceptibility to lysis, the cultures were grown on agar and transplanted only as often as the experiments required. Whenever resistant bacteria were needed for the test, a subculture from agar was made into broth and used as such after 18 hours of incubation at 37°C.

Virulence of the Resistants by Feeding.

After having carried the resistant strains W-Little R_7 and W-178 R_7 for four more generations on agar (eleven in all) we attempted to determine their virulence as compared with that of the original strain of *B. pestis caviæ*.

Protocol II.

Virulence of Resistants by Feeding.

Bacteria fed		Group A (control) Stock M. T. II	Group B W-Little R_{11}	Group C W-178 R_{11}
No. of bacteria given		3,000,000	3,000,00	3,000,000
No. of mice surviving by days	5th day	25	25	25
	6th "	23	25	25
	7th "	19	25	25
	8th "	15	25	25
	9th "	12	25	25
	10th "	10	25	25
	11th "	9	25	25
	12th "	8	25	25
	13th "	8	25	25
	14th "	8	25	25

The animals used for these experiments came from special stock kept at The Rockefeller Institute, and known to have been free from spontaneous mouse typhoid for a period of years. It was hoped that these animals would show no heightened resistance to the infection, and would present an ideal object for the study to be undertaken.

75 mice of approximately 25 gm. weight each were divided into three equal groups. To each mouse of the first group (Protocol II, *A*) was given, by means of a stomach tube, 0.5 cc. of an 18 hour broth culture of *B. pestis caviæ* (M. T. II), diluted to contain about 3,000,000 bacteria in each dose. The mice of the second group (Protocol II, *B*) each received about 3,000,000 bacteria (in 0.5 cc. volume)

from an 18 hour broth subculture from the eleventh generation of a resistant W-Little R_{11} on agar. The mice of the third group (Protocol II, *C*) each received 0.5 cc. of broth containing about 3,000,000 bacteria of an 18 hour broth subculture of W-178 R_{11}. Each animal was placed in an individual jar, its ordinary ration was supplied daily, and the litter was changed three times per week. No symptoms were noted until the 4th or 5th day after the feeding of bacteria, when certain animals in Group A appeared ill, and from the 6th day deaths began to occur, as indicated on Protocol II. The experiment was interrupted on the 15th day, after no deaths had occurred among the animals for 3 days. At this time all the animals receiving resistant bacteria (Groups B and C) were living, whereas only eight animals out of twenty-five (about 32 per cent) survived the feeding of the original M. T. II.

Susceptibility to Infection by B. pestis caviæ (M. T. II) of Mice Surviving the Feeding of Resistants.

In the next two experiments the resistants of the thirteenth and fourteenth generations on agar were used. The method was the same as in the first experiment and the results may be summarized by stating that the mortality of controls (corresponding to Group A on Protocol II) was 62 and 66 per cent respectively, whereas all the animals but two of those fed the resistants remained alive. That these two deaths did not arise from mouse typhoid infection is rendered probable by the fact that at the autopsy the blood and internal organs were found to be free from *B. pestis caviæ*.

Since the failure of resistants to infect mice was contrary to what was expected on the basis of earlier reports in the literature (1–5, 11), it was thought advisable to ascertain the susceptibility of the surviving mice in the above experiments to infection with the parent culture M. T. II; if these animals prove susceptible to subsequent infection with M. T. II, their earlier resistance to W-Little R_{14} and W-178 R_{14} respectively can be ascribed to a change in virulence in these cultures.

Accordingly, twenty mice each of those (twenty-five) surviving feeding with W-Little R_{14} and W-178 R_{14} respectively (corresponding to Groups B and C on Protocol II) were divided into two groups. The first subgroup of ten mice from each group received a suspension of an 18 hour broth culture of virulent M. T. II by mouth, the other by intraperitoneal injection. Similarly, half of twenty normal mice were given the same bacterial suspension by mouth, and the other half by intraperitoneal injection. The animals were cared for exactly as in the earlier ex-

periments, except that those injected intraperitoneally were placed in larger containers—five mice in each. Protocol III illustrates the results obtained.

The remaining eight mice surviving the feeding of resistants[2] were killed and examined for signs of invasion by the bacteria which had been fed to them. As in the case of two mice of this series which died

Protocol III.

Susceptibility of Surviving Mice to Infection by M. T. II.

		Mice surviving the feeding of resistants 15 days previously				Normal controls	
		Survivals of Group B (fed W-Little R_{14})		Survivals of Group C (fed W-178 R_{14})			
No. of mice		10	10	10	10	10	10
Mode of infection		By mouth	Intraperitoneal	By mouth	Intraperitoneal	By mouth	Intraperitoneal
No. of virulent bacteria given (about)		5,000,000	2,000,000	5,000,000	2,000,000	5,000,000	2,000,000
No. of deaths per day	1st day		3		4		3
	2nd "		3		3		6
	3rd "		1		3		1
	4th "		2				
	5th "		1			1	
	6th "			1		2	
	7th "	2		3			
	8th "	1				1	
	9th "	3		1		2	
	10th "						
	11th "	1					
	12th "			1		1	
	13th "						
	14th "						
No. of survivals		3	0	4	0	3	0

earlier, the internal organs of these mice were sterile, but the intestinal contents showed the presence of bacteria resembling the parent strain M. T. II in their lack of ability to ferment lactose, in the production of H_2S, and in their immunologic properties. They differed

[2] Twenty mice of each series of twenty-five were used in the preceding experiment, and one animal in each group died from intercurrent cause, thus leaving four mice in each group (see experiment Protocol II).

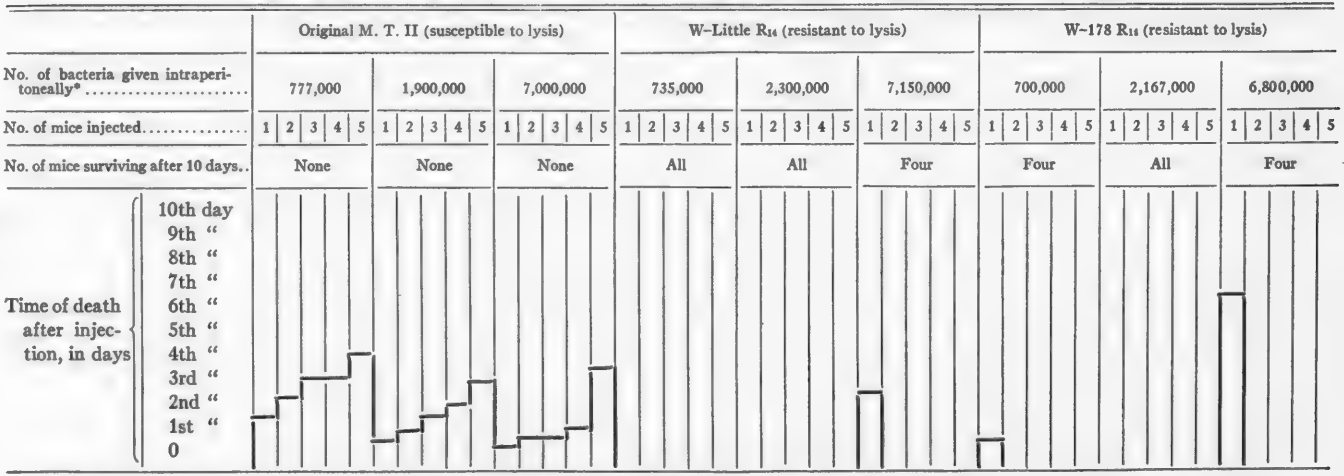
only in their power to resist lysis by homologous lytic agents, thus establishing their identity with the material fed to the mice. In other experiments of similar nature, the intestinal contents yielded an occasional colony susceptible to lysis; the bulk of bacteria fed to the mice seemed, however, to have remained in the intestinal contents without becoming susceptible to lysis, and without entering into the blood for at least 14 days. If this be proven to be the case with larger experimental material, it would suggest the loss of invasive power by the resistants.

Virulence of Resistants by Intraperitoneal Injection.

The fact that mice surviving the feeding of resistants were still susceptible to subsequent infection by the virulent parent M. T. II strain, together with the finding that the resistants lack invasive power, would indicate that their failure to kill animals in earlier experiments was due to this change in virulence, as suspected. However, we thought it of interest to inquire whether this change in virulence was limited to their loss of invasive power only, or to some more radical change. For this reason we compared their power to infect mice by parenteral route with that of the parent strain M. T. II. Accordingly, three series of fifteen mice each were given, by intraperitoneal injection, varying doses of the original M. T. II, W-Little R_{14}, and W-178 R_{14} respectively, as indicated on Protocol IV, and the time of death of each mouse was noted. It will be seen from this protocol that whereas all the mice receiving the original M. T. II injection were dead before the expiration of 5 days (after injection), only three mice out of a total of thirty animals injected with resistants were dead up to the 10th day, when the experiment was terminated. We are inclined to attribute the death of these three mice to other causes rather than to the infection, particularly since in two subsequent experiments analogous to the one just described—except that the dose of bacteria was doubled and trebled respectively—only one mouse died, out of a total of 60 receiving resistant bacteria intraperitoneally, whereas the mortality of mice receiving the original M. T. II culture was invariably 100 per cent. These experiments, in our opinion, indicate that at least in the case of *B. pestis caviæ* isolation of cultures resistant to lysis by bacteriophage results in obtaining an avirulent strain of this organism.

Protocol IV.

The Loss of Virulence by the Resistant Subcultures of M. T. II as Tested by Intraperitoneal Injection.

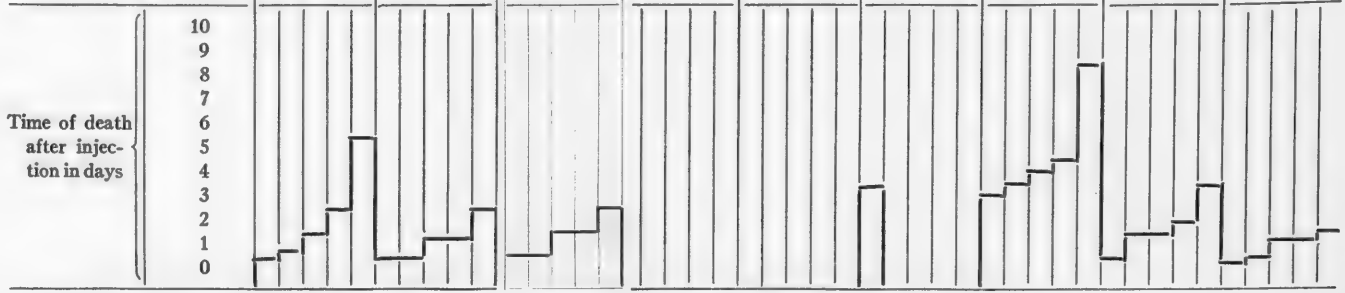


* These figures show the bacterial count on agar plates poured immediately after injection of animals.

Protocol V.

Virulence of the Reverted W-Little R_{14} by Intraperitoneal Injection.

	A			B			C		
				W-Little R_{14} (resistant variant)					
	Original culture of M. T. II			Grown for 18 hrs. on broth			After 20 daily passages on broth		
Character of growth on broth	Diffuse			Agglutinated			Diffuse		
Susceptibility to lysis by lytic agent W–Little	Susceptible			Not susceptible			Susceptible		
No. of bacteria given intraperitoneally*	966,000	2,900,000	8,700,000	1,366,000	4,100,000	12,300,000	967,000	3,100,000	9,700,000
No. of mice injected	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
No. of mice surviving after 10 days (virulence)	None	None	None	All	All	Four	None	None	None



* These counts were obtained by plating samples of suspensions of bacteria immediately after injection.

Virulence of W-Little R_{14} after Its Recovery of Susceptibility to Lysis.

It is well known that bacteria isolated from the overgrowth of lysed cultures usually revert to the original type and become susceptible to lysis after a more or less prolonged cultivation on media free from lytic agent. Since the preceding experiments indicated that there may exist an interdependence between the lack of susceptibility to lysis and the loss of virulence in the culture of *B. pestis caviæ*, it seemed desirable to find out whether the return of susceptibility to lysis will be followed by a return of virulence in such cultures.

For this purpose the fourteenth generation of a resistant strain (W-Little R_{14}) was subcultured from agar into broth, and from the latter daily transfers to broth were continued for 20 days, after which the culture thus obtained was tested for its susceptibility to lysis, as well as for its virulence to mice, by the intraperitoneal route. For the sake of comparison parallel tests were made with the culture of the original M. T. II, as well as with an 18 hour broth culture obtained by inoculating into sterile broth a small loop from the old agar slant W-Little R_{14}, which presumably retained its resistance to lysis by the phage W-Little. The procedure followed in this experiment was entirely analogous to that of the preceding experiment (Protocol IV), except that in addition to other tests, each culture was subjected to a control test of its susceptibility to lysis. The results of the virulence test are given in Protocol V. Similarly, the twentieth passage in broth from W-Little R_{14} proved capable of killing mice when given by mouth (Protocol VI).

It is evident, then, that when full reversion to susceptibility to lysis, after prolonged cultivation in broth and in the absence of bacteriophage, has taken place, the resistant strain W-Little R_{14}, which remains avirulent and resistant to lysis on the first subculture in broth (Protocols V, *B* and VI, *B*), tends to become as virulent as the parent culture of M. T. II, from which it originally was derived.[3]

In order to ascertain more closely the relationship between the return of virulence and the susceptibility to lysis of the culture, the experiment was repeated and the test of susceptibility, as well as of virulence, was made daily.

[3] It should be noted incidentally that simultaneously with these changes in virulence and susceptibility to lysis, the appearance of growth on broth changed from being agglutinated in the first subculture from agar to the diffuse growth characteristic of the parent culture M. T. II.

Protocol VI.

Virulence of the Reverted W-Little R_{14} by Mouth.

	A	B	C
	Original M. T. II	W–Little R_{14} (resistant to lysis)	
	18 hr. culture on broth	Grown 18 hrs. on broth	Subjected to 20 passages on broth
Approximate No. of bacteria given by mouth in .5 cc. volume	5,000,000	5,000,000	5,000,000
No. of mice	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10	1 2 3 4 5 6 7 8 9 10
Time of death after feeding in days: 14th day			
13th "			
12th "			
11th "			
10th "			
9th "			
8th "			
7th "			
6th "			
5th "			
4th "			
3rd "			
2nd "			
1st "			
No. of survivals	3	All 10	5

For this purpose we returned to the agar culture W-Little R_{14} (kept on ice all the time), and made a subculture from it into broth. After 18 hours of incubation at 37°C. the resulting broth culture was tested for its virulence and susceptibility to lysis. A little later on the same day, a second subculture in broth was made from the first to be tested next day (after 18 hours of growth) and so on for several days (Protocol VII).

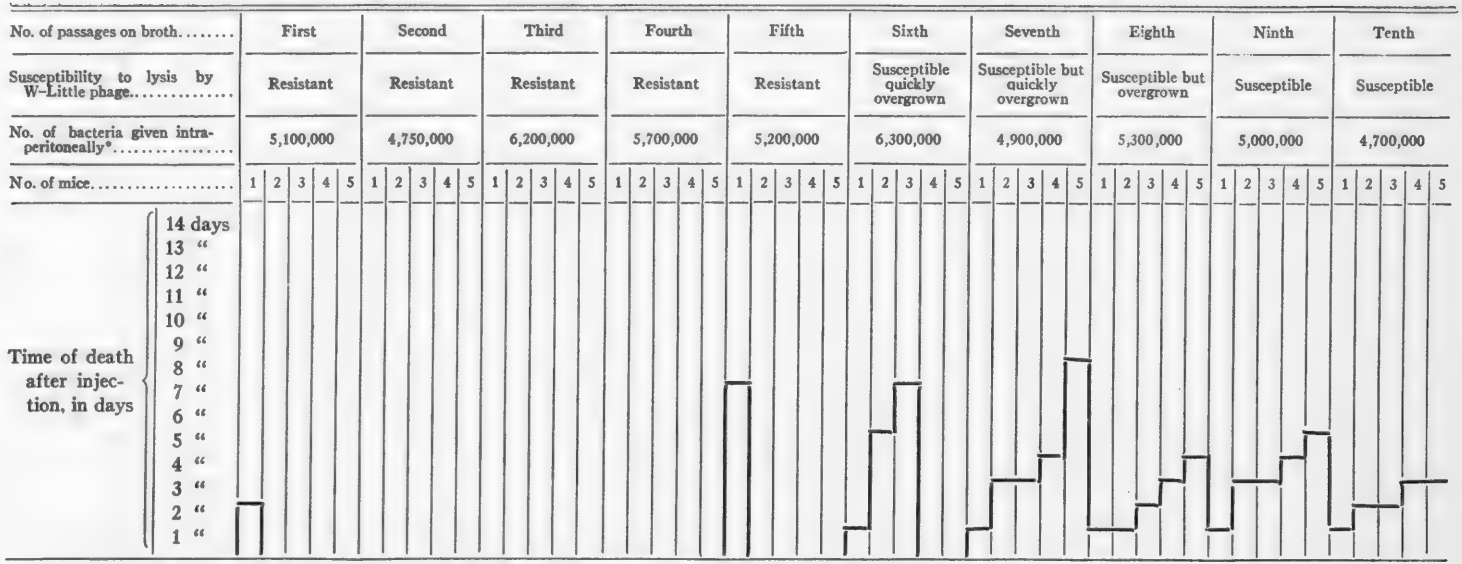
Only the intraperitoneal method of injection was used for testing virulence in this experiment, as it was deemed more convenient, both because of simplicity and the shorter incubation period. When it was observed that the animals died regularly, at a rate approximating that of the mortality of mice injected with the original M. T. II culture, the experiment was interrupted. Results of this experiment indicate that on the sixth transfer in broth the resistant culture W-Little R_{14} became susceptible to lysis, and at the same time it regained its pathogenicity.

Non-Reversion to Susceptibility of W-178 R_{14}.

As stated in the early part of this paper, resistant strains obtained by us could be roughly divided into two groups, judging by the macroscopic appearance of their respective growth in broth. Since it has been observed further that resistants secured by means of the phage W-Little are susceptible to lysis by the phage W-178 (but not *vice versa*, see Protocol I), we undertook to ascertain whether resistants obtained by the action of the latter "stronger" phage would also undergo reversion to susceptibility and recover virulence if grown in the absence of phage. However, all attempts in this direction have thus far been unsuccessful. The cultures have remained resistant to lysis, and when tested for virulence, after nearly 200 successive passages in broth over a period of 10 months, mice survived an injection of 3,000,000 bacteria intraperitoneally. Throughout the period resistant bacteria exhibited their original characteristics as regards fermentation and antigenic properties, and were found to be free from bacteriophage (not "lysogenic"). Occasionally, when grown on agar plates with a corresponding bacteriophage, some of these cultures gave rise to a few "pale" plaques which were quickly overgrown by resistants. In such cases corresponding broth cultures have, on occasion, shown a slight increase in the titer of the phage, but at

Protocol VII.

Respective Rate of Recovery of Virulence and of Susceptibility to Lysis by the Resistant Subcultures of W-Little R_{14}.



* The numbers recorded above were obtained by plating suspensions immediately after injection.

no time was it possible to isolate the few susceptible individuals which must have appeared temporarily in such cultures, to be immediately overgrown by the resistants or to be destroyed by the phage. Since our first observations concerning the behavior of resistants obtained

Protocol VIII.

Attempt to Cause Reversion in the Presence of Antiphage Serum.

No. of transfers previous to test	Culture medium	Character of growth	Susceptibility to lysis as tested	
			In broth	On 1 per cent agar
1	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
2	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
4	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
6	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
7	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
10	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
17	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
25	Plain broth	Diffuse	—	—
	Broth + serum	Sediment and diffuse overgrowth	—	—
32	Plain broth	Diffuse	—	—
	Broth + serum	Sediment and diffuse overgrowth	—	—

by means of bacteriophage W-178 (18), we have noted a similar failure of resistants to revert to susceptibility when other "strong" phages were used for their production. Although in every instance the resistants were tested and found not to carry phage, we attempted

to grow them in the presence of an antibacteriophage serum, in the hope that we might thus induce a reversion.

In these experiments a resistant strain, isolated by means of a phage P.I.D. and carried for several months without reversion, was employed. Starting from an agar slant culture of this strain P.I.D.-R_{15}, we have made two sets of daily transfers, the first into plain broth and the second into broth containing 0.02 cc. of antibacteriophage serum for each cc. of the medium. From time to time cultures of resistants thus obtained were tested for their susceptibility to lysis in broth as well as in agar. This procedure was continued for a month without leading to reversion, as illustrated on Protocol VIII.

DISCUSSION.

According to the original conception of d'Hérelle and his collaborators, the production of resistants is the result of an increase in the resistance of bacteria against the invasion of bacteriophage. They consider this process analogous to the development of active immunity in higher forms, after exposure to infection (19). In accordance with this theory, resistants might be expected to be more virulent, and actual observation seems to have confirmed the expectations in many instances (2–7). However, our experiments fail to support this notion since, at least in the case of *B. pestis caviæ*, resistants have been found repeatedly to be devoid of virulence. On the other hand, only cultures susceptible to lysis have been found to be pathogenic. In a measure, as cultures of resistants recover their susceptibility to lysis, they again become virulent. The cultures which fail to become susceptible to lysis, under the conditions of the experiment, remain avirulent. The fact that some resistant strains may not become susceptible, after having been carried free from bacteriophage for 200 successive transfers in broth, seems to indicate that the production of resistants is not a phenomenon of active "hereditary" immunization (12), but rather a result of irreversible variation, somewhat analogous to that observed in pneumococcus cultures (13, 14). It has been shown by Arkwright (15), Gratia (16), and others that cultures of bacteria of the colon-typhoid group can be normally dissociated into a number of variants of which at least two are manifestly different in the appearance of their colonies. Gratia (17) found later that the variants normally possess different resistance

to lytic agents and may exhibit varying rates of growth (3). Thus, a bacterial culture may be considered in its cross-section (as indeed any other "population") as composed of individuals approaching a certain type, but occasionally lacking or taking on exaggerated characteristics. Depending upon the conditions of growth, some of the variants may find themselves favored by environment and may become quantitatively dominant. On the contrary, if the environment is changed so that it becomes incompatible with life or the multiplication of a certain type of variant, the latter is eliminated more or less completely, and the whole cross-section of the bacterial population in the culture changes accordingly, with more or less noticeable changes in the biologic activity of the culture as a whole. In the case of a highly specific, "weak" bacteriophage which displays activity only toward a comparatively narrowly defined type of individuals in the culture (as, for example, the phage W-Little), only the most susceptible individuals carrying the potential characteristic of virulence are destroyed, and if such a culture is allowed to grow in the absence of bacteriophage, the few remaining closely related individuals, which may carry the potential characteristic of virulence, begin to multiply anew and to produce the original cross-section of the culture. If, however, a phage of less specificity is used (as, for example, the "strong" phages W-178 or P.I.D.), it may happen that all the individuals carrying the characteristic of virulence are destroyed, and a permanently avirulent culture results.

SUMMARY AND CONCLUSIONS.

Resistants isolated from the overgrowth of cultures of *B. pestis caviæ* (M. T. II) lysed by various strains of specific bacteriophage proved to be avirulent when administered to mice by feeding, or by intraperitoneal injection.

These cultures remained resistant to the action of bacteriophage so long as they were carried on agar. When transferred to broth, however, one group of resistants, namely, those isolated by means of "weak" phages, became susceptible to lysis after five to seven daily passages. The other group of resistants, isolated from the cultures lysed by one of the "strong" phages, failed to become susceptible to lysis even after nearly 200 passages in broth.

Simultaneously with the recovery of susceptibility, the cultures of the first group regained a degree of virulence comparable to that of the parent culture of *B. pestis caviæ*. The cultures of the second group of resistants have failed thus far to recover virulence (10 months after isolation). The latter cultures, apart from lack of both virulence and susceptibility to lysis, are identical with the parent culture of *B. pestis caviæ*, as indicated by biochemical and antigenic properties.

Our findings offer evidence in favor of the view that resistant strains result from selection among variants already existing in the parent culture and do not arise through the inheritance of specific immunity properties produced by the action of phage.

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THE SURVIVAL OF THE TUBERCLE BACILLUS IN SUSPENSION IN PHYSIOLOGICAL SALT SOLUTION.

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The length of time that tubercle bacilli will live and maintain virulence when suspended in physiological salt solution has never been finally determined. The matter is of interest to those who use saline suspensions of the organisms in producing experimental infections. Webb, Ryder, and Gilbert (1) have noted that a saline suspension of tubercle bacilli was able to produce tuberculosis in guinea pigs after it had stood for as long as 87 days. They did not test its virulence for longer periods of time.

This note is to place on record the observation that three suspensions of tubercle bacilli in physiological saline were alive and virulent for guinea pigs after standing for periods of 310, 325, and 330 days, respectively, at refrigerator temperature.

The refrigerator used fluctuates about the freezing point, the fluctuations not being recorded. The suspensions were made by lightly grinding the moist bacilli from active glycerol agar cultures in an agate mortar, adding 0.85 per cent NaCl solution slowly at first and then more rapidly to the amount of 1 cc. per mg. of bacilli. The suspensions were stored in narrow neck, round flasks of pyrex glass stoppered with cotton. Evaporation was not measured but was slight in amount.

Two of these suspensions were of the same strain of organism, Bovine 18, and the other suspension was of a human type, 7156. Two guinea pigs were used in determining the viability of each suspension, one receiving 0.1 mg. of organisms intraperitoneally, and the second 1 mg. in like manner.

The results are presented in Table I.

All of these animals showed a well marked generalized tuberculosis and there was nothing atypical in the course of the disease or in the autopsy findings.

Culture Bovine 18 has not been used recently in infecting guinea pigs and consequently it is impossible to state how much of its virulence has been lost during its prolonged suspension in salt solution. Culture H 7156 has, however, recently been so used. In a group of 29 guinea pigs each received 0.1 mg. of H 7156 subcutaneously. The average length of life for the group was 164 days, with the extreme variations at 88 and 230 days. The animal receiving 0.1 mg. of the 325 day old suspension of H 7156 intraperitoneally died in 145 days. It would seem then that the virulence has not been greatly diminished.

TABLE I.

Culture	Age of suspension	Dose	Length of life, in days, following infection
	days	*mg.*	
18	310	0.1	93
18	310	1.0	160
18	330	0.1	98
18	330	1.0	128
7156	325	0.1	145
7156	325	1.0	108

SUMMARY.

Three suspensions of tubercle bacilli in physiological salt solution were still virulent for guinea pigs after 310, 325, and 330 days at refrigerator temperature. One of these cultures on which recent tests had been made had lost very little, if any, of its virulence for guinea pigs in 325 days.

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THE BEHAVIOR IN VIVO OF CERTAIN RELATIVELY PURE ANTIGENS.

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The fact that certain proteins, such as those of blood serum, when introduced into the body will persist in the circulation for considerable periods is well known. It is also true that in certain individuals precipitin will appear in the blood before the antigen has disappeared. Thus the paradox of antigen and antibody occurring in the blood at the same time is frequently encountered. Several explanations of this phenomenon have been offered. Some have favored the view that an excess of colloid will inhibit the combination of antibody and antigen. Others have pointed out that such substances as egg white and serum are composed of several proteins and that the precipitin which appears in the blood may really be due to a single antigen which has been exhausted during the production of the antibody. The resulting antibody would react with a portion of the complex antigen and the remaining proteins would still be capable of reacting with the complex precipitin.

During the last few years the view that the paradox is explicable on the basis of multiple antigens has come into considerable prominence although in the main relatively few experiments have been reported to support this view. Those of Bayne-Jones[1] cannot be said to do so. He was able to show that rabbits immunized with crystallized egg albumen, or edestin, until their serum contained precipitin in considerable concentration would on reinjection of the specific antigen contain both antigen and antibody for a period of 48 hours. He further brought out the fact that *in vitro* much the same phenomenon was observed when antigen and antibody were mixed and the resulting precipitin centrifuged out. The supernatant fluid contained both antibody and antigen. Previously, however, R. Weil's[2] experiment indicated that this phenomenon held true only when multiple

[1] Bayne-Jones, S., *J. Exp. Med.*, 1917, xxv, 837.

[2] Weil, R., *J. Immunol.*, 1916, i, 19.

antigens were employed. Thus when horse serum or egg white was mixed with its respective antiserum and the precipitate removed by centrifugation, the supernatant liquid contained both antigen and antibody. With crystalline egg albumen the results were not the same. In these experiments he showed that both antigen and antibody never occurred together in the supernatant liquid. The fact that the presence of a third colloid failed to interfere with the interaction between antigen and antibody was demonstrated in his experiments.

The work of Opie[3] follows more closely the phenomena subsequent to the therapeutic administration of horse serum. From his first series of experiments it is evident that after injection of horse serum into the blood stream of rabbits, precipitin for horse serum appears in the blood before the horse serum proteins entirely disappear. This he attributes to the composite antigenic nature of the horse serum, and suggests that certain of the contained proteins give rise to antibodies much more rapidly than certain others. When similar experiments were performed with a relatively pure crystallized egg albumen the findings were different. It is clear from Opie's findings that when crystallized egg albumen is employed as antigen the corresponding antibody and uncombined egg albumen do not appear in the blood together.

Ascoli[4] had called attention to the fact that after the injection of egg white it appeared in the urine. Opie[5] also found that crystallized egg albumen appeared in the urine subsequent to injection. Therefore, if such be the case, the injection of crystallized egg albumen hardly affords a parallel to the administration of foreign blood proteins. It is well known that the administration of large amounts of horse serum for therapeutic purposes does not result in the appearance of the foreign serum proteins in the urine provided the kidney is intact. The writer has on several occasions injected 5 or 6 cc. of cow serum into the peritoneal cavity of normal rabbits but in no instance has it been possible to detect it in the urine, although its presence in the rabbit's blood serum could readily be determined.

It seemed necessary to study the behavior of crystallized egg albumen after its injection to determine whether its rapid elimination might not afford an explanation for the fact that antibody failed to appear while the antigen was still present. The experiments indicate that the behavior of egg albumen is in certain respects different from that of foreign blood proteins, so that in the second series of observations casein, an antigen which on the whole affords an analogy to the blood proteins in its general behavior, was used.

[3] Opie, E. L., *J. Immunol.*, 1923, viii, 55.
[4] Ascoli, M., *Münch. med. Woch.*, 1902, xlix, 398.
[5] Opie, E. L., *J. Exp. Med.*, 1924, xxxix, 659.

EXPERIMENTAL.

Rabbits, 2,500 gm. in weight, were immunized with various antigens and after sera of sufficient titer were obtained they were bled and the serum stored in the refrigerator. The specific precipitin was used to test the blood serum and urine for the presence of antigen. The test rabbits were of about the same age and size. The injections were made intravenous or intraperitoneal and the animals bled at frequent intervals from the ear vein. The blood was collected in sterile test-tubes and permitted to clot in a thin layer, in the incubator, and when sufficient serum had oozed out it was collected and centrifuged. In this way a clear serum was available for the tests within 2 or 3 hours.

The naturally voided urine was collected in clean bottles. It was filtered twice through filter paper.

TABLE I.

The Results of the Tests for the Presence of Antigen in the Blood Serum of Rabbit 1 Injected Intravenously with Crystallized Egg Albumen.

Time after last injection that sample was taken	Amount of serum tested				
	1.0 cc.	0.5 cc.	0.2 cc.	0.1 cc.	0.05 cc.
18 hrs.	−*	−	−	−	−
42 "	−	−	−	−	−
3 days	−	−	−	−	−
4 "	−	−	−	−	−

* Since crystallized egg albumen reacts powerfully with its antibody, the intensity of the reaction has been recorded as follows: +++, a heavy precipitate; ++, a moderate precipitate; +, slight but definite precipitation; ±, turbidity with a trace of precipitate.

The crystallized egg albumen was prepared by precipitation with ammonium sulfate after the method of Hopkins and Pinkus.[6] It was recrystallized four times. For the casein I am indebted to Dr. J. H. Northrop of The Rockefeller Institute. It was prepared by his method.[7]

Experiment 1.—Rabbit 1. Weight, 2,245 gm. On Nov. 2, 1925, at 9.45 a.m., received 3 cc. of a 5 per cent solution of crystallized egg albumen intravenously. The animal urinated at 4.10 p.m. At 4.45 p.m., 2.5 cc. of the egg albumen was again injected into the ear vein. A blood sample was taken on Nov. 3, at 10 a.m. Blood for the tests was drawn daily and the urine was collected during the next

[6] Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898–99, xxiii, 130.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 749.

few days. The results of the tests of blood for both egg albumen and antibody are given in Tables I and II, and those of the urine in Table III.

TABLE II.

The Development of Antibody in the Blood of Rabbit 1 Subsequent to Intravenous Injection of Crystallized Egg Albumen.

Time after injection tests were made	Dilutions of antigen					
	1:40	1:80	1:160	1:320	1:640	1:1,280
days						
1	−	−	−	−	−	−
2	−	−	−	−	−	−
3	−	−	−	−	−	−
4	−	−	−	−	−	−
6	−	−	−	−	−	−
8	−	−	−	−	−	−
9	−	−	−	−	−	−
10	±	±	±	±	±	±
11	+	+	+	+	+	+

TABLE III.

The Appearance of Crystallized Egg Albumen in the Urine of Rabbit 1 Injected Intravenously with Crystallized Egg Albumen.

Urine voided	Dilutions of urine tested										
	1:0	1:1	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
6½ hrs. after 1st injection	−	+	++	+++	+++	+++	++	+	+	±	−
During night following injection (from 2 to 16 hrs. after 2nd injection)	−	−	+	++	+++	+++	+++	+	+	±	−
During 2nd night following injection	+	±	−	−	−	−	−	−	−	−	−
46 hrs. after 2nd injection	−	−	−	−	−	−	−	−	−	−	−
During 3rd night following injection	−	−	−	−	−	−	−	−	−	−	−

It becomes apparent that Opie's contention that crystalline egg albumen and its respective precipitin are not demonstrable together in the blood is well founded. From the results obtained in this experiment there may be some doubt that the crystallized egg

albumen precipitin was really active when used to test the rabbit's serum. It, however, was capable of reacting in the animal's urine. However, the contention that crystalline egg albumen differs in its behavior from the blood proteins of a foreign species when introduced into the body is true. From the tables it is clear that the antigen rapidly disappears from the circulation, since it could not be detected 18 hours following injection. That it rapidly passes through the kidney and in relatively large quantities is also true, since it was present in high dilutions of urine (1/640 cc.) as early as 6½ hours after its injection. Its elimination is rapid, since it only ap-

TABLE IV.

Examination of the Blood Serum of Rabbit 2 for Egg Albumen at Various Intervals after Its Injection.

Time after first injection	Dilutions of serum tested (in cc.)*							
	1:0	1:1	1:5	1:10	1:20	1:40	1:80	1:160
hrs.								
1	±	+	++	++	+	+	±	−
2	+	++	+	+	+	−	−	−
3	++	+	+	+	±	−	−	−
4	+	+	−	−	−	−	−	−
6	+	±	−	−	−	−	−	−
7	±	±	−	−	−	−	−	−
19†	−	−	−	−	−	−	−	−
43†	−	−	−	−	−	−	−	−

* In the tests of the 2, 3, 4, and 6 hour samples, 0.7, 0.75, 0.65, and 0.8 cc., respectively, were used instead of 1 cc. as indicated.

† After 2nd injection.

peared in the urine approximately 30 hours after intravenous administration. After this time it could no longer be detected. Definite antibody appeared in the blood on the 10th day following injection.

Since in the first experiment the antigen had not been detected in the circulation, it might be said that the precipitin was unable to react in the presence of an excess of colloids. To meet this objection, and as a general confirmation of the first observation, the following experiment was performed.

Experiment 2.—Rabbit 2, weighing 2,405 gm., was injected intravenously, at 9.30 a.m. with 3 cc. of 5 per cent crystalline egg albumen. An hour later it was bled and at intervals throughout the day. Urine was passed 5½ hours after the first injection. At 4.45 p.m., 3 cc. of the albumen solution was injected into the ear vein. As in the previous experiment, the blood was regularly tested for egg albumen and its antibody. The urine was also tested for antigen. Inasmuch as the experiment added little to the development of precipitin, the details will not be given. It is sufficient to state that antibody appeared in the blood on the 7th day. The results of the tests of the blood serum and urine for egg albumen are recorded in Tables IV and V.

TABLE V.

Examination of the Urine of Rabbit 2 for Egg Albumen after Its Intravenous Injection.

Time urine was voided	Dilutions of urine tested							
	1:0	1:1	1:5	1:10	1:20	1:40	1:80	1:160
5½ hrs. after 1st injection	−	−	±	++	++	++	+	+
During night following 2nd injection (from 2 to 16 hrs. after 2nd injection)	±	±	+	+	++	++	+	±
18 hrs. after 2nd injection	±	±	±	±	±	−	−	−
During 2nd night following 2nd injection	−	−	−	−	−	−	−	−
63 hrs. after 2nd injection	−	−	−	−	−	−	−	−
72 " " " "	−	−						
4 days " " "	−	−						

The findings in the second experiment fully confirm those of the first. The contention that crystallized egg albumen is rapidly eliminated from the circulation is well borne out. It can be readily detected in the blood during the first 3 hours following its administration, but by the end of the 4th hour it has decreased considerably. After 7 hours only a trace remains and after 18 hours it can no longer be detected. The precipitin used in the observations would react with as little as 1/700,000 gm. of the antigen. The passage of the albumen through the kidney compares in a rough way with the decline in the circulation. In both instances the urine contained the antigen in considerable concentration as early as 6 hours after its injection. Egg albumen could be demonstrated only for a period of 1 or at most 2 days in the urine. The specific antibody appeared in the blood in one instance in 7 and in the other in 10 days after the injection of the antigen.

Since crystallized egg albumen fails to meet the general requirements, the number of relatively pure proteins of general availability is somewhat limited. Bayne-Jones used with considerable success

TABLE VI.

The Behavior of Casein and Its Antibody in Rabbits 3 and 4.

Rabbit No.	Days after injection	Test of serum for casein in		Test of urine for casein in			Test of serum for antibody			
							Dilutions of antigen			
		1.0 cc.	0.1 cc.	1.0 cc.	0.5 cc.	0.1 cc.	1:100	1:200	1:400	1:800
3	1	+*	−	−	−	−	−	−	−	
	2	+	−	−	−	−	−	−	−	
	3	+	±	−	−	−	−	−	−	
	4	+	±	−	−	−	−	−	−	
	6	+	?	−	−	−	−	−	−	
	7	+	?				−	±	±	
	8	+	?				+	+	+	
	9	+	−				+	+	+	
	10	+	−				+	+	+	
	11	±	−				±	+	+	+
	13	±	−				±	+	+	+
	14	±	−				±	+	+	+
	16	−	−					±	+	+
4	1			−	−					
	2			−	−					
	3	+		−	−		−	−	−	
	4	+		−	−		−	−	−	
	5	+		−	−		−	−	−	
	7	+					−	−	−	
	8	+					−	−	−	
	9	+					±	±	±	
	10	±					±	+	+	
	11	±					+	+	+	
	12	±					+	+	+	
	14	−					+	+	+	

* + indicates a well defined turbidity; ±, a slight turbidity.

the globulin edestin. In my hands edestin proved unsatisfactory. When dissolved in sufficiently strong alkali it could not be injected with safety. If by titration one attempted to reduce the free alkali it would give a clear solution, but on the addition of small amounts

of normal rabbit serum a ring would form at the point of contact between the two liquids and after mixing precipitation would occur. Its use for this reason was discontinued.

Gay and Robertson[8] and Wells and Osborne[9] showed that purified casein is antigenic. By the repeated intraperitoneal injection of a 1 per cent solution of casein a precipitin was obtained which would in amounts of 0.1 or 0.2 cc. react with as little as 1/100,000 gm. of casein. As a rule the distinct line at the union of serum and casein occurred promptly. In the higher dilutions there is little actual precipitation but the mixed liquids promptly become turbid.

That casein, on the whole, meets the requirements of a relatively pure protein which will persist in the circulation for a considerable period is brought out in the following observations.

Experiment 3.—On different occasions rabbits were injected with casein. The casein was dissolved in N/20 NaOH and sterile salt solution. The solution was injected intraperitoneally in 3 doses during the day. Rabbit 3 received 0.6 gm. of casein; Rabbit 4 0.5 gm.

All the voided urine was collected and tested during the next 5 days. The blood serum from both rabbits was tested at frequent intervals for casein with the anticasein serum. As a control procedure tubes of the experimental animals' sera were incubated and refrigerated. In this way an accurate comparison of turbidity was possible. The urine was twice filtered through filter paper and tested in the same manner. Control tubes of the same sample were always incubated and refrigerated. The tests for casein precipitin were carried out in the usual manner. 0.2 cc. of the rabbit's serum was added to 1 cc. of the various dilutions of the antigen. The results are given in Table VI.

It is apparent that casein, like the blood proteins, remains in the circulation for considerable periods. Even as long as 12 and 13 days after its injection the serum responds weakly to the specific test. Like the serum proteins it fails to pass through the kidney in quantities sufficient to be detected. In both instances antibody was recognized in the blood serum while the casein was still in the circulation.

DISCUSSION AND SUMMARY.

The experiments are of interest in several respects. It is clear that crystallized egg albumen is rapidly eliminated from the circula-

[8] Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1912, xvi, 470.
[9] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1921, xxix, 200.

tion and in the experiments cited it could no longer be detected after 18 or 19 hours. A considerable portion of it rapidly passes through the kidney in an apparently unaltered state. Evidently this passage begins almost at once and may continue for a day or two. In an experiment not reported in this paper, egg albumen appeared in naturally voided urine 2 hours following its injection into the peritoneal cavity. In the experiments reported no urine was voided until $5\frac{1}{2}$ and $6\frac{1}{2}$ hours following intravenous administration, but in each instance egg albumen was present in considerable amounts. However, sufficient egg albumen must have been utilized to produce antibody. It is hardly to be expected that such a protein, whose elimination is so rapid, could persist unaltered within the body and reappear within the circulation coincident with its antibody. The behavior of the protein cannot be ascribed to alterations which may have taken place during the process of crystallization since Ascoli showed that the proteins of egg white readily pass from the circulation into the urine. Certain observations of the writer confirm this point. The experience of Alexander, Becke, and Holmes[10] who exposed sensitized guinea pigs to sprays of dilute egg white with the result that 80 per cent of the animals developed symptoms of anaphylaxis, further strengthens the contention that certain of the membranes are readily permeable for the proteins of egg.

The conditions following the injection of casein are different. There is no appreciable passage through the kidney. Casein is present within the circulation for a considerable period; it could be detected in the blood serum 12 and 13 days after its introduction into the peritoneal cavity. Antibody appeared on the 7th and 8th days, respectively, so that both antigen and antibody were present in the serum for a period of 3 or 4 days. The phenomenon of antigen and antibody occurring together might be explained on the ground that certain proteins are utilized slowly and that the antibody found in the blood, usually after the 7th day, results from the portion of antigen first utilized. During the next few days a continual supply of antibody enters the circulation and during the period there is a steady utilization of the antigenic substance; it is possible that during

[10] Alexander, H. L., Becke, W. G., and Holmes, J. A., *J. Immunol.*, 1926, xi, 175.

this time there is constant union of antigen and antibody within the blood, with the slow utilization of the antigen and a slight utilization of the antibody which is made up by a slow increase from the body cells. Thus there would be a period in which considerable antigen would be present with weak antibody, succeeded by a second period when the amount of antigen would be small with well defined antibody, and finally only antibody. Certain observations tend to support such a view. Bayne-Jones injected rabbits whose serum contained precipitin from egg albumen with this substance and noted the occurrence of both antigen and antibody for a period of 48 hours. Some of his experiments *in vitro* are equally suggestive. In one instance a rabbit well immunized with egg albumen was injected intravenously with this substance. An hour later it was bled and the stored serum refrigerated for a period. During this time there was a slow spontaneous precipitation with a decline in both precipitin and antigen titer, but even after 6 days both were present. After a longer period only antigen remained. P. A. Lewis and D. Loomis[11] have shown that an injection of sheep red blood cells in guinea pigs results in a well defined hemolysin titer about the 9th day, followed by a definite decline, with a secondary rise in hemolysin until the peak is reached on the 20th day.

It becomes evident, then, that the reaction of the rabbit to a single injection of a relatively pure protein will depend on the character of the protein injected. When crystallized egg albumen is administered it is rapidly eliminated from the circulation. The rapid disappearance of the egg albumen from the blood stream is partly accounted for by its prompt elimination through the urine. Antibody appears in the serum from the 7th to the 10th day. Casein behaves differently. It persists in the blood for a considerable period; after the 7th or 8th day both antigen and antibody may be demonstrated in the blood. Casein cannot be detected in the urine following its injection into the body. The behavior of casein within the body affords an analogy with the conditions frequently noted after the administration of foreign serum, in both cases both antigen and antibody may be present in the circulation together.

[11] Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1924, xl, 503.

THE RELATION BETWEEN BODY AND ORGAN WEIGHTS IN THE RABBIT.

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In the previous paper of this series (1), we summarized the results of that part of our study of organ weights the immediate object of which was to define conditions that obtain in normal stock rabbits with respect to mean weights, the tendency to the occurrence of variations in weight, and the probable limits of variation for different organs. As a further contribution to the general problem of physical constitution and as an approach to the study of conditions that determine or affect the weights of organs, we have undertaken an investigation of the normal relation that obtains between body and organ weight and between the weight of one organ and that of another. The purpose of this paper is to report the relation found between body and organ weight as indicated by correlation coefficients and a comparison of group means.

Methods and Material.

The results to be reported are based on data from 645 male rabbits of various breeds. The series contained a few young and a few old animals but the great majority of the animals were between 6 months and 2 years old with about an equal division between those that were sexually mature but not full grown and those that had attained sexual maturity and full growth. All of the animals were in apparent good health but on postmortem examination many of them showed active or healed lesions of some kind. In the present investigation no discrimination was made on the basis of age, breed, or the presence of lesions.

The actual weight and the weight of organs per kilo of net body weight[1] (relative

[1] Net body weight is the gross weight of the animal minus the weight of the gastrointestinal mass as defined in the first paper of this series (Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1925, xlii, 69).

TABLE I.

Correlation Coefficients for Actual and Relative Organ Weight with Gross Body Weight.

	Actual	Relative
Net body weight	+0.958 ±0.002	
Heart	+0.755 ±0.011	−0.169 ±0.026
Testicles	+0.555 ±0.019	+0.101 ±0.028
Kidneys	+0.471 ±0.021	−0.443 ±0.021
Gastrointestinal mass	+0.456 ±0.021	−0.311 ±0.024
Brain	+0.451 ±0.025	−0.761 ±0.013
Suprarenals	+0.364 ±0.023	−0.066 ±0.027
Hypophysis	+0.343 ±0.024	−0.402 ±0.022
Mesenteric lymph nodes	+0.307 ±0.03	−0.143 ±0.032
Liver	+0.291 ±0.024	−0.301 ±0.024
Thyroid	+0.248 ±0.025	−0.019 ±0.027
Deep cervical lymph nodes	+0.246 ±0.031	−0.058 ±0.033
Thymus	+0.222 ±0.025	−0.160 ±0.026
Parathyroids	+0.221 ±0.025	−0.174 ±0.026
Pineal	+0.193 ±0.026	−0.316 ±0.024
Popliteal lymph nodes	+0.190 ±0.028	−0.271 ±0.027
Spleen	+0.186 ±0.026	−0.113 ±0.026
Axillary lymph nodes	+0.074 ±0.029	−0.321 ±0.026

TABLE II.

Correlation Coefficients for Actual and Relative Organ Weight with Net Body Weight.

	Actual	Relative
Gross body weight	+0.937 ±0.003	
Heart	+0.739 ±0.012	−0.217 ±0.025
Testicles	+0.493 ±0.021	+0.018 ±0.028
Kidneys	+0.452 ±0.021	−0.473 ±0.021
Suprarenals	+0.415 ±0.022	+0.027 ±0.027
Brain	+0.392 ±0.027	−0.830 ±0.010
Hypophysis	+0.353 ±0.023	−0.449 ±0.021
Thyroid	+0.261 ±0.025	+0.013 ±0.027
Mesenteric lymph nodes	+0.251 ±0.031	−0.222 ±0.031
Gastrointestinal mass	+0.245 ±0.025	−0.448 ±0.021
Parathyroids	+0.242 ±0.025	−0.139 ±0.026
Deep cervical lymph nodes	+0.228 ±0.032	−0.103 ±0.033
Liver	+0.214 ±0.025	−0.417 ±0.022
Thymus	+0.213 ±0.025	−0.171 ±0.026
Pineal	+0.199 ±0.026	−0.323 ±0.024
Spleen	+0.143 ±0.025	−0.166 ±0.026
Popliteal lymph nodes	+0.116 ±0.029	−0.290 ±0.027
Axillary lymph nodes	+0.063 ±0.029	−0.336 ±0.026

TABLE III.

Group Means for Body (Gross) and Organ Weights.

Gross body weight	No. of animals	Net body weight	Heart		Testicles		Kidneys		Gastrointestinal mass		Brain	
			Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1.400	1	1175	3.95	3.36	3.22	2.74	20.29	17.28	225.0	191.5		
1.563	2	1245	3.53	2.91	3.74	3.06	11.04	8.87	317.5	261.7	9.38	8.16
1.633	6	1306	3.57	2.74	2.27	1.74	10.44	7.97	327.5	252.9	8.18	6.32
1.750	20	1415	4.08	2.89	3.09	2.20	11.17	7.90	335.7	245.9	8.44	6.04
1.840	36	1472	4.27	2.90	3.80	2.53	11.76	7.99	365.1	250.6	8.60	5.80
1.933	56	1553	4.62	2.98	3.74	2.41	11.98	7.73	380.0	244.4	8.87	5.69
2.033	89	1629	4.86	2.99	4.15	2.53	12.20	7.52	401.6	248.7	8.86	5.46
2.134	83	1717	5.02	2.93	4.16	2.45	12.46	7.26	415.2	243.6	9.23	5.39
2.235	78	1811	5.11	2.81	4.72	2.61	12.71	7.04	420.0	233.9	9.28	5.12
2.338	75	1908	5.37	2.82	5.02	2.60	13.01	6.84	439.0	225.7	9.26	4.87
2.436	59	2005	5.73	2.87	4.99	2.48	13.77	6.88	431.1	217.2	9.27	4.63
2.531	44	2070	5.71	2.76	5.58	2.70	13.68	6.62	457.3	222.2	9.43	4.54
2.638	23	2160	5.94	2.74	5.29	2.40	13.67	6.32	474.3	220.6	9.24	4.29
2.732	25	2272	6.42	2.71	5.87	2.58	14.28	6.27	445.4	197.1	9.76	4.27
2.815	13	2282	6.35	2.79	6.19	2.73	14.43	6.33	527.3	233.0	9.82	4.30
2.936	11	2440	6.85	2.82	6.52	2.66	16.44	6.74	485.0	200.3	9.65	3.91
3.047	9	2562	7.03	2.75	6.43	2.52	15.53	6.11	485.2	187.5	10.06	3.95
3.107	7	2605	7.12	2.97	6.33	2.69	15.81	6.65	497.0	191.9	9.39	4.09
3.200	2	2530	7.45	2.94			16.20	6.41	670.0	265.0	11.30	4.51
3.350	4	2855	7.74	2.72	7.30	2.69	16.48	5.82	470.0	166.3	10.28	3.66
3.450	1	2925	7.20	2.46	8.58	2.93	15.10	5.16	525.0	179.5	11.03	3.77
3.500	1	3010	7.68	2.55	8.98	2.98	15.78	5.23	490.0	162.8		
Correlation coefficient			+0.958 ±0.002	+0.755 ±0.011	−0.169 ±0.026	+0.555 ±0.019	+0.101 ±0.028	+0.471 ±0.021	−0.443 ±0.021	+0.456 ±0.021	−0.311 ±0.024	+0.451 ±0.025	−0.761 ±0.013

TABLE III—*Concluded.*

Gross body weight	No. of animals	Suprarenals		Hypophysis		Mesenteric lymph nodes		Liver		Thyroid		Deep cervical lymph nodes	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1.400	1	0.228	0.194	0.030	0.0255			55.0	46.8	0.175	0.149		
1.563	2	0.505	0.3955	0.0255	0.0208			83.0	68.9	0.1625	0.1311		
1.633	6	0.275	0.2097	0.0237	0.0175	3.03	2.38	61.3	46.8	0.1725	0.1321	0.0988	0.0772
1 750	20	0.3222	0.2259	0.0264	0.0187	2.67	1.92	75.9	54.1	0.2864	0.2024	0.1114	0.0782
1.840	36	0.3285	0.2204	0.0259	0.0176	2.48	1.67	76.5	52.2	0.1866	0.1263	0.1252	0.084
1.933	56	0.3238	0.2083	0.0253	0.0164	3.18	2.05	80.1	51.7	0.1897	0.1216	0.1414	0.0914
2.033	89	0.3239	0.1984	0.0269	0.0166	3.34	2.06	82.3	50.6	0.1944	0.1194	0.1558	0.0959
2.134	83	0.3339	0.1944	0.0273	0.0159	3.26	1.91	82.0	48.0	0.217	0.1266	0.1458	0.0858
2.235	78	0.3694	0.2038	0.0272	0.0150	3.70	2.04	83.4	47.0	0.2079	0.1149	0.1428	0.0785
2.338	75	0.4226	0.2203	0.0282	0.0148	3.38	1.79	92.5	47.4	0.2269	0.1186	0.1484	0.0787
2.436	59	0.4332	0.2164	0.0307	0.0154	3.55	1.79	89.6	44.9	0.2468	0.1221	0.1629	0.0809
2.531	44	0.4358	0.2104	0.0301	0.0142	3.61	1.75	91.0	44.1	0.3095	0.1467	0.1758	0.0851
2.638	23	0.4239	0.1964	0.0311	0.0144	3.95	1.84	84.0	43.7	0.2507	0.1154	0.1678	0.0781
2.732	25	0.4436	0.1947	0.0301	0.0133	4.09	1.79	91.2	40.2	0.2835	0.1239	0.2024	0.0884
2.815	13	0.4117	0.1801	0.0314	0.0138	3.82	1.68	96.9	42.8	0.3202	0.1394	0.2511	0.1108
2.936	11	0.5868	0.2405	0.0319	0.0130	4.19	1.69	92.0	37.8	0.3905	0.1587	0.1969	0.0796
3.047	9	0.5177	0.2006	0.0315	0.0123	4.03	1.57	93.8	36.8	0.2611	0.1019	0.1979	0.0777
3.107	7	0.7000	0.2996	0.0343	0.0142	4.65	1.80	101.6	39.1	0.355	0.1509	0.1238	0.0598
3.200	2	0.475	0.1877	0.0365	0.0144	8.10	3.23	94.5	37.4	0.2075	0.0821	0.185	0.0739
3.350	4	0.436	0.1517	0.0302	0.0106	3.83	1.36	114.8	40.6	0.3775	0.1343	0.2083	0.0753
3.450	1	0.635	0.217	0.032	0.0109	4.00	1.37	120.0	41.0	0.436	0.149	0.225	0.0769
3.500	1	0.590	0.1967	0.030	0.0100	4.85	1.61	90.0	29.9	0.235	0.0784	0.125	0.0416
Correlation coefficient		+0.364	−0.066	+0.343	−0.402	+0.307	−0.143	+0.291	−0.301	+0.248	−0.019	+0.246	−0.058
		±0.023	±0.027	±0.024	±0.022	±0.03	±0.032	±0.024	±0.024	±0.025	±0.027	±0.031	±0.033

Gross body weight	No. of animals	Thymus		Parathyroids		Pineal gland		Popliteal lymph nodes		Spleen		Axillary lymph nodes	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1.400	1	1.280	1.090	0.010	0.00851	0.015	0.01276	0.180	0.1532	1.15	0.978	0.150	0.1276
1.563	2	2.103	1.720	0.015	0.01213	0.0135	0.011	0.230	0.1715	1.165	0.915	0.130	0.097
1.633	6	1.553	1.197	0.0115	0.00887	0.0163	0.01253	0.184	0.1421	0.787	0.603	0.115	0.0891
1.750	20	1.889	1.334	0.0136	0.00952	0.0144	0.01016	0.208	0.1473	0.753	0.528	0.1497	0.1063
1.840	36	1.991	1.291	0.01161	0.00791	0.0153	0.01041	0.232	0.1546	0.925	0.629	0.1667	0.1138
1.933	56	2.110	1.356	0.01141	0.00735	0.0148	0.0095	0.243	0.1565	0.943	0.602	0.1592	0.1019
2.033	89	2.234	1.375	0.01208	0.00744	0.0152	0.00931	0.252	0.1552	0.954	0.590	0.1687	0.1034
2.134	83	2.269	1.334	0.01197	0.00699	0.0155	0.00906	0.256	0.1492	1.042	0.609	0.1646	0.09597
2.235	78	2.240	1.236	0.0119	0.00661	0.0151	0.00828	0.261	0.1435	0.930	0.519	0.1824	0.1034
2.338	75	2.317	1.224	0.01343	0.00705	0.0158	0.00828	0.261	0.1377	1.044	0.550	0.1739	0.0915
2.436	59	2.356	1.175	0.01338	0.00671	0.0159	0.00793	0.263	0.1312	1.061	0.533	0.1805	0.0896
2.531	44	2.527	1.221	0.01495	0.00726	0.0179	0.00871	0.258	0.1254	1.170	0.572	0.1714	0.0832
2.638	23	2.740	1.272	0.01308	0.00607	0.0161	0.00749	0.285	0.1319	1.013	0.469	0.1794	0.0831
2.732	25	2.657	1.172	0.01356	0.00598	0.0163	0.00716	0.250	0.1100	1.181	0.522	0.164	0.0721
2.815	13	2.689	1.181	0.01346	0.00593	0.0155	0.00683	0.272	0.1200	1.249	0.552	0.1646	0.0734
2.936	11	2.599	1.066	0.01454	0.00593	0.0155	0.00632	0.256	0.1039	1.195	0.489	0.1511	0.0614
3.047	9	2.626	1.026	0.01511	0.00592	0.0197	0.00771	0.304	0.1198	1.246	0.487	0.1661	0.0653
3.107	7	2.739	1.054	0.02143	0.00917	0.021	0.00809	0.279	0.1052	1.502	0.574	0.173	0.0664
3.200	2	2.350	0.928	0.017	0.00671	0.022	0.00871	0.250	0.0999	1.895	0.749	0.175	0.0695
3.350	4	2.530	1.094	0.01625	0.00564	0.0205	0.00716	0.338	0.1195	0.995	0.350	0.2217	0.0776
3.450	1	3.100	1.060	0.012	0.0041	0.018	0.00616	0.320	0.1094	1.300	0.445	0.215	0.0735
3.500	1	1.890	0.630	0.032	0.01064	0.025	0.00834	0.470	0.1561	0.930	0.309	0.180	0.0598
Correlation coefficient		+0.222 ±0.025	−0.160 ±0.026	+0.221 ±0.025	−0.174 ±0.026	+0.193 ±0.026	−0.316 ±0.024	+0.190 ±0.028	−0.271 ±0.027	+0.186 ±0.026	−0.113 ±0.026	+0.074 ±0.029	−0.321 ±0.026

TABLE IV.

Group Means for Body (Net) and Organ Weights.

Net body weight	No. of animals	Gross body weight	Heart		Testicles		Kidneys		Suprarenals		Brain	
			Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1.163	2	1475	3.89	3.34	3.82	3.30	15.26	13.09	0.264	0.228	9.38	8.16
1.247	4	1681	3.93	3.15	2.94	2.37	10.20	7.91	0.288	0.223	8.34	6.69
1.358	14	1733	3.81	2.80	2.62	1.92	11.14	8.19	0.326	0.240	8.53	6.26
1.443	38	1827	4.33	3.0	4.89	3.34	11.42	8.17	0.308	0.211	8.72	6.06
1.550	87	1964	4.66	3.0	3.92	2.52	12.09	7.81	0.313	0.202	8.85	5.70
1.650	90	2067	4.87	2.95	4.21	2.52	12.62	7.66	0.328	0.200	9.07	5.50
1.745	79	2151	4.98	2.85	4.09	2.34	12.34	6.93	0.349	0.200	8.96	5.13
1.844	87	2260	5.24	2.83	4.96	2.69	13.05	7.07	0.372	0.202	9.46	5.11
1.952	70	2382	5.46	2.79	5.14	2.64	13.02	6.69	0.451	0.232	9.18	4.70
2.046	59	2470	5.72	2.80	5.07	2.45	13.51	6.60	0.434	0.212	9.24	4.53
2.146	44	2596	5.86	2.73	5.48	2.54	13.79	6.42	0.427	0.197	9.41	4.38
2.243	23	2729	6.41	2.85	5.78	2.60	14.10	6.47	0.414	0.185	9.58	4.28
2.350	13	2796	6.86	2.93	6.48	2.76	14.66	6.25	0.559	0.238	9.66	4.10
2.461	12	2945	6.97	2.83	6.72	2.73	16.12	6.53	0.448	0.182	9.95	4.04
2.544	9	3044	6.51	2.77	6.34	2.77	15.55	6.56	0.583	0.254	10.01	4.45
2.655	9	3110	7.11	2.68	6.59	2.48	16.13	6.11	0.681	0.257	9.65	3.62
2.870	1	3300	7.34	2.56	9.12	3.15	18.13	6.31	0.475	0.166	9.70	3.38
2.942	3	3392	7.47	2.54	7.56	2.74	14.30	4.85	0.540	0.183	10.62	3.62
3.010	1	3500	7.68	2.55	8.98	2.98	15.78	5.23	0.590	0.197		
Correlation coefficient			+0.937	+0.739	−0.217	+0.493	+0.018	+0.452	−0.473	+0.415	+0.027	+0.392	−0.830
			±0.003	±0.012	±0.025	±0.021	±0.028	±0.021	±0.021	±0.022	±0.027	±0.027	+0.010

Net body weight	No. of animals	Hypophysis		Thyroid		Mesenteric lymph nodes		Gastrointestinal mass		Parathyroids	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1.163	2	0.029	0.02492	0.1675	0.144			312.0	269.8	0.0125	0.01073
1.247	4	0.0245	0.01858	0.1513	0.1217	3.42	2.73	434.0	348.9	0.0113	0.009
1.358	14	0.0251	0.01849	0.1712	0.1264	2.38	1.74	375.0	276.1	0.0104	0.0077
1.443	38	0.0258	0.01791	0.2343	0.1632	2.97	2.06	381.0	267.2	0.0128	0.0089
1.550	87	0.0254	0.01682	0.185	0.1195	3.20	2.07	412.0	264.8	0.0112	0.0072
1.650	90	0.0274	0.01662	0.2101	0.1273	3.33	2.02	413.0	250.5	0.0129	0.0079
1.745	79	0.0269	0.01544	0.2089	0.1199	3.39	1.94	405.0	232.3	0.0118	0.0068
1.844	87	0.0278	0.01511	0.2096	0.1139	3.53	1.91	414.0	225.1	0.0122	0.0066
1.952	70	0.0286	0.01465	0.2262	0.1151	3.53	1.81	425.0	217.7	0.0137	0.0071
2.046	59	0.0306	0.01496	0.2688	0.131	3.31	1.62	423.0	206.8	0.014	0.0069
2.146	44	0.0303	0.01418	0.3021	0.1406	3.60	1.68	448.0	208.8	0.0139	0.0065
2.243	23	0.0291	0.01299	0.2723	0.1213	4.33	1.93	475.0	211.8	0.012	0.0054
2.350	13	0.031	0.0133	0.2943	0.1679	4.18	1.69	432.0	184.0	0.0125	0.0053
2.461	12	0.0324	0.01318	0.3977	0.1332	4.24	1.72	478.0	192.2	0.0165	0.0067
2.544	9	0.0335	0.0139	0.3733	0.1579	4.42	1.74	504.0	198.2	0.0172	0.0075
2.655	9	0.033	0.01244	0.3272	0.1232	4.14	1.56	448.0	168.7	0.0179	0.0064
2.870	1	0.035	0.0122	0.490	0.1716	2.72	0.95	430.0	149.8	0.016	0.0055
2.942	3	0.030	0.0102	0.3137	0.1067	4.60	1.56	450.0	153.0	0.0166	0.0057
3.010	1	0.030	0.00997	0.235	0.0784	4.85	1.61	490.0	162.8	0.032	0.0106
Correlation coefficient		+0.353 ±0.023	−0.449 ±0.021	+0.261 ±0.025	+0.013 ±0.027	+0.251 ±0.031	−0.222 ±0.031	+0.245 ±0.025	−0.448 ±0.021	+0.242 ±0.025	−0.139 ±0.026

TABLE IV—*Concluded.*

Net body weight	No. of animals	Deep cervical lymph nodes		Liver		Thymus		Pineal gland		Spleen	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
kg.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1.163	2			83.5	72.1	1.867	1.613	0.0150	0.0129	0.940	0.806
1.247	4	0.0938	0.0752	74.8	59.5	1.345	1.073	0.0155	0.0124	0.753	0.601
1.358	14	0.1008	0.0745	72.2	53.1	1.744	1.285	0.0138	0.0102	0.848	0.640
1.443	38	0.1285	0.0891	81.2	56.3	2.014	1.392	0.0144	0.0100	0.886	0.613
1.550	87	0.1392	0.090	82.4	53.1	2.024	1.310	0.0144	0.0093	0.955	0.620
1.650	90	0.164	0.0996	83.2	50.3	2.268	1.381	0.0158	0.0096	1.021	0.616
1.745	79	0.1447	0.0833	80.8	46.3	2.245	1.291	0.0159	0.0092	1.022	0.588
1.844	87	0.1437	0.0779	88.2	47.6	2.279	1.242	0.0153	0.0083	0.963	0.525
1.952	70	0.152	0.0777	87.3	44.7	2.334	1.196	0.0161	0.0082	1.053	0.539
2.046	59	0.161	0.0787	89.5	43.8	2.587	1.267	0.0166	0.0081	1.100	0.535
2.146	44	0.1754	0.0817	91.2	42.5	2.526	1.175	0.0164	0.0077	1.140	0.530
2.243	23	0.2196	0.0981	96.0	42.6	2.816	1.255	0.0153	0.0069	1.030	0.458
2.350	13	0.1781	0.0756	86.9	36.9	2.514	1.070	0.0166	0.0071	1.078	0.458
2.461	12	0.2205	0.0895	90.2	36.6	2.648	1.077	0.0188	0.0077	1.058	0.423
2.544	9	0.166	0.0656	90.5	35.6	2.285	0.897	0.0189	0.0074	1.334	0.525
2.655	9	0.1933	0.0726	96.3	36.3	2.282	1.153	0.0184	0.0069	1.551	0.586
2.870	1	0.150	0.0527	115.0	40.1	2.050	0.714	0.0200	0.0070	0.900	0.313
2.942	3	0.1875	0.0639	106.7	36.3	2.763	0.940	0.0210	0.0071	1.093	0.372
3.010	1	0.125	0.0416	90.0	29.9	1.890	0.630	0.0250	0.0083	0.930	0.309
Correlation coefficient		+0.228 ±0.032	−0.103 ±0.033	+0.214 ±0.025	−0.417 ±0.022	+0.213 ±0.025	−0.171 ±0.026	+0.199 ±0.026	−0.323 ±0.024	+0.143 ±0.025	−0.166 ±0.026

Net body weight	No. of animals	Popliteal lymph nodes		Axillary lymph nodes	
		Actual	Relative	Actual	Relative
kg.		*gm.*	*gm.*	*gm.*	*gm.*
1.163	2	0.180	0.1532	0.150	0.1276
1.247	4	0.1725	0.1377	0.1388	0.1109
1.358	14	0.2418	0.1769	0.1527	0.1118
1.443	38	0.2311	0.1569	0.1607	0.1111
1.550	87	0.239	0.1551	0.1568	0.101
1.650	90	0.2546	0.1543	0.1705	0.1033
1.745	79	0.2583	0.1483	0.1659	0.0951
1.844	87	0.2639	0.143	0.1813	0.0984
1.952	70	0.2577	0.1319	0.1756	0.0897
2.046	59	0.2738	0.134	0.1845	0.090
2.146	44	0.2439	0.1135	0.1563	0.0728
2.243	23	0.2264	0.1232	0.1733	0.0773
2.350	13	0.2512	0.1068	0.160	0.0679
2.461	12	0.2845	0.1154	0.1702	0.0694
2.544	9	0.2225	0.0878	0.1292	0.0511
2.655	9	0.2681	0.0997	0.1584	0.0595
2.870	1	0.350	0.122	0.180	0.0627
2.942	3	0.3475	0.1182	0.2775	0.0945
3.010	1	0.470	0.1561	0.180	0.0598
Correlation coefficient		+0.116 ±0.029	−0.290 ±0.027	+0.063 ±0 029	−0.336 ±0.026

weight) were correlated with both gross and net body weight. The correlation coefficients are recorded in Tables I and II in the order of the magnitude of the coefficients obtained for actual weight. Space does not permit the publication of complete correlation tables but a summary of the group means is given in Tables III and IV arranged on the basis of increasing gross and net body weights respectively. The results for gross body weight are plotted in Text-fig. 1; those for net body weight do not differ sufficiently to warrant reproduction. In order to facilitate direct comparison the values for organ weight are plotted on the basis of a percentage deviation from the mean weight of the organ concerned for a given increase in body weight so that the scales of all curves are comparable. The significant parts of the curves are included between heavy perpendicular lines. The groups to the right or left of these lines contained only a few animals but the values are given as they were used in calculating the coefficients.

RESULTS.

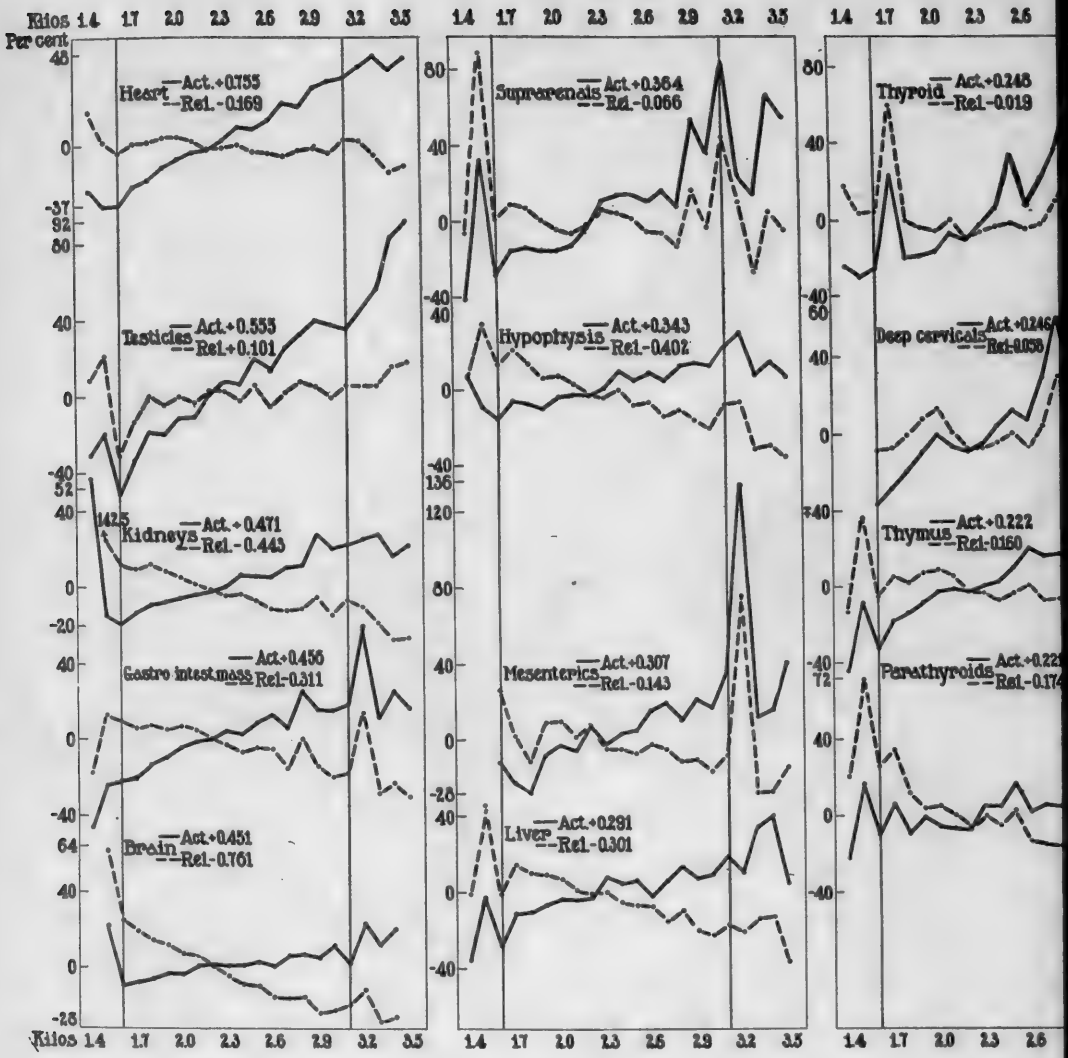
The relations found between body and organ weights are presented in Tables I to IV and Text-fig. 1.

DISCUSSION AND CONCLUSIONS.

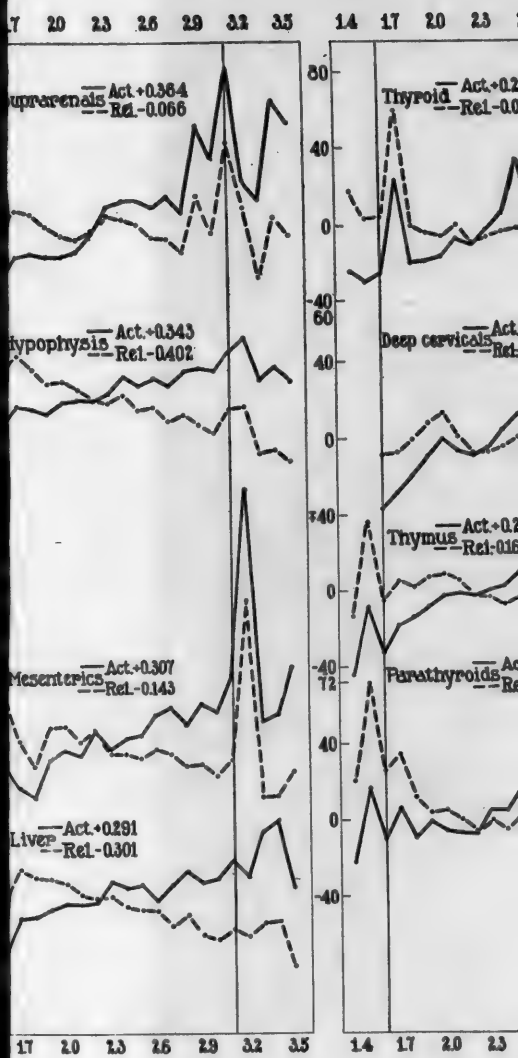
The results presented in Tables I to IV and Text-fig. 1 bring out a number of important points concerning the physical constitution of mature and apparently healthy stock rabbits. With very few exceptions, the ratio of the correlation coefficients to their probable errors (Tables I and II) is sufficiently large to warrant acceptance of the results as valid measures of the relation existing between body and organ weight, irrespective of the magnitude of the coefficients.

The coefficients show a great diversity of relations. In the first place, it will be seen that there is a *positive* relation of some kind between the actual weight of all organs and the body weight of the animal while the coefficients for relative weight are either *negative* or *approach a zero order*. That is to say, there is evidence of a common tendency on the part of all organs to weigh more or less according to the weight of the animal but, in only a few instances, is the difference in weight of such an order as to maintain a constant relation between body and organ weight; the weight of the organ per kilo of body weight varies and, as a rule, diminishes as the weight of the animal increases.

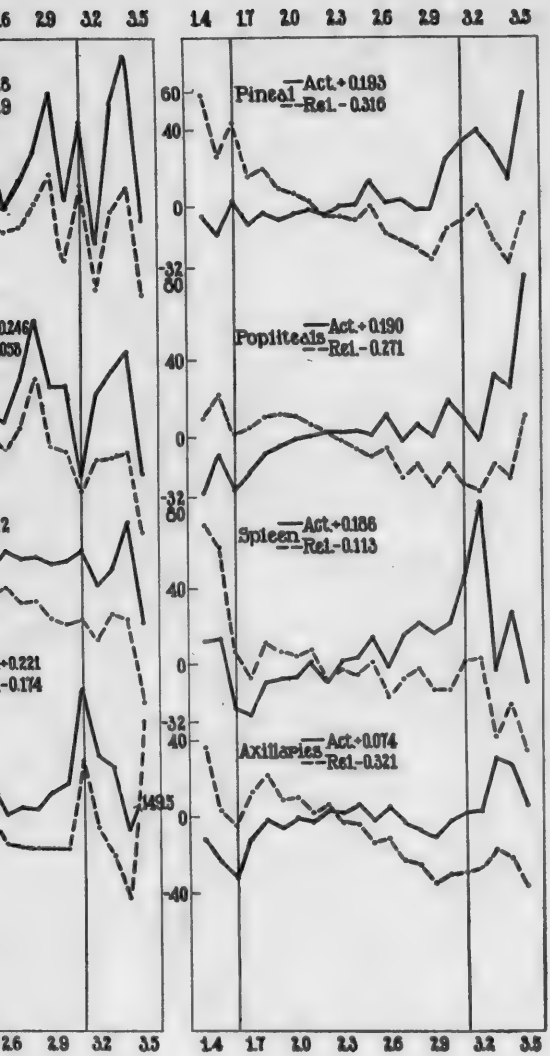
In estimating the degree of the correlation there are two values to



TEXT-FIG. 1. Relation of actual and relative organ weights to body weight plotted as a percentage deviation values for all animals. (Act. = actual organ weight, Rel. = relative organ weight.)



gan weights to body weight plotted as a percentage dev
, Rel. = relative organ weight.)



ation of the group means for organ weight from the mean

be considered, first, the direct correlation between the actual weight of the organ and that of the body and, second, the inverse relation between the weight of the organ per kilo of body weight and the weight of the body. By reference to Tables I and II, it will be seen that there is no constant relation between these two values. The ideal condition of a high positive and a low negative coefficient, or a coefficient of the zero order, is shown by very few organs. In some cases this relation is reversed while in others the two sets of coefficients are of a comparable magnitude. If, however, we arrange the organs according to the magnitude of the correlation coefficients for actual weight with gross body weight, as in Table I, it will be seen that in general the so called major organs of the body show the highest values and the lymphoid organs the lowest with the endocrine glands occupying an intermediate position.

The situation presented by correlating relative organ weight with either gross or net body weight is entirely different. The coefficient obtained in this way serves as an inverse measure of the extent to which the increase in the actual weight of different organs approximates the ideal condition of the maintenance of a constant ratio between body and organ weight. In most instances the magnitude of the coefficient for relative weight is smaller than that for actual weight.

Similar conditions obtain when the weights of organs are correlated with net instead of gross body weight (Table II). The chief difference between the two sets of results is in the magnitude of the correlation coefficients for different classes of organs. By correlating actual organ weight with net body weight, the coefficients for the endocrine glands are increased while those for all other organs are either diminished or unaffected; in the case of relative weights, all values are increased with the exception of those for the thyroid, parathyroids, suprarenals, and testicles. While in most instances the change in the magnitude of the coefficient is comparatively small, the effect of this method of correlation is to strengthen the direct correlation of the endocrine glands and to weaken that of other organs.

A clearer conception of the form as well as the measure of the relation between body and organ weight may be gained by an examination of the curves in Text-fig. 1 which give the results obtained by

plotting the group means for actual and for relative organ weight against the corresponding values for gross body weight (Table III). These curves show three distinct forms of relation: first, a linear increase in actual weight which is directly proportional to body weight; second, a straight line increase in actual weight which is of such an order as to produce a linear decrease in the weight of the organ per kilo of body weight; third, an increase in the actual weight of the organ over the lower ranges of body weight with the maintenance of a constant or diminishing level of actual weight over the middle and upper ranges which produces first an increase and then a decrease in the weight of the organ per kilo of body weight (popliteal and axillary lymph nodes).

Other curves appear to be modifications or combinations of these with the possible exception of the curve for the suprarenals. There is some indication that the change in the weight of the suprarenals is not of the order of a uniform progression but shows a diphasic condition characterized by stabilization of actual weights at successive levels or by a succession of increases and decreases in relative weight. It may be that this condition is referable to some factor other than body weight which, in the case of the suprarenals, is sufficiently potent to obscure the influence of the body weight factor.

Analyzing the results obtained from the standpoint of the correlation coefficients and the form of the relation shown, it will be seen that there are comparatively few organs the weights of which are closely related to body weight. The heart shows the closest correlation from every point of view. The coefficient for actual weight is much larger than that of any other organ while the coefficient for relative weight is comparatively small. This, in itself, suggests that there is a constant ratio between the weight of the heart and the weight of the body which holds for practically all ranges of weight within the limits of these observations. This conclusion is borne out by plotting the mean values for actual and relative weights (Table III) against body weight. The curve obtained (Text-fig. 1) shows a linear increase in the actual weight of the heart which is of such an order as to maintain the weight per kilo at a practically constant level. This may be taken as an example of almost perfect physical correlation between the weight of an organ which performs a mechanical function and that of the body which it serves.

The testicles show a similar relation to body weight, giving a high correlation coefficient for actual weight and a small positive coefficient for relative weight, while the mean values (Table III) form a curve which shows the same relation between actual and relative weights on the one hand, and the weight of the body on the other, as the curve for the heart. This result may be regarded as highly significant and indicates that the generally recognized relation between testicular development and growth is of a very high order.

Among the organs studied, the testicles and the heart are the only ones that show a high correlation of this type. The kidneys, the gastrointestinal mass, and the brain come next in the order of magnitude of the correlation coefficients between actual and gross body weight. But, these organs show a negative coefficient for relative weight of a high order which would lead one to infer that the rate of increase in the actual weight of the organ is not proportional to the increase in body weight. By plotting the mean values (Table III, Text-fig. 1), we find that, within the limits of these observations, the increase in the weight of the kidneys is fairly uniform but the rate of increase is such that the weight per kilo of body weight diminishes at a rate which is approximately equal to that of the increase in actual weight, giving correlation coefficients of essentially the same magnitude ($+0.471 \pm 0.021$ and -0.443 ± 0.021). The gastrointestinal mass behaves in much the same manner but the coefficient for relative weight is smaller ($+0.456 \pm 0.021$ and -0.311 ± 0.024), and the regression in the weight of the mass per kilo of body weight is correspondingly less.

The coefficients for the liver ($+0.291 \pm 0.024$; -0.301 ± 0.024) are of a lower order than those for the kidneys and gastrointestinal mass but they show a similar relationship to body weight with a suggestion of a tendency toward an accentuation of the inverse relation which is brought out so strikingly in the case of the brain. The condition presented by the brain is the reverse of that shown by the organs mentioned above. The coefficient for actual weight is $+0.451 \pm 0.025$ while that for relative weight is -0.761 ± 0.013. These values would lead one to expect a very uniform but very small increase in the actual weight of the brain with increasing body weight. The curve formed by the group means (Table III; Text-fig. 1) shows

a slight and very uniform increase in the actual weight of the brain for animals weighing between 1500 and 2000 gm.; from 2000 to 2500 gm. it is doubtful whether there is any change but in still heavier animals there is apparently a second increase of the same order but less uniform than the first.

The organs considered above form a small group with comparatively close and clearly defined relations to body weight. At the opposite end of the scale, we find such purely lymphoid organs as the popliteal and axillary lymph nodes with negative coefficients that are distinctly larger than the positive coefficients for actual weight. The relation shown by these organs is of a comparatively low order and even this may be an effect of age rather than weight.

It will be seen that the mesenteric and deep cervical lymph nodes differ from the popliteal and axillary nodes; the coefficients for actual weight are larger and the negative coefficients are smaller so that the relation of these two masses of lymphoid tissue to body weight is not only closer than that of the superficial lymph nodes but is also of a different character (Text-fig. 1).

The conditions shown by the spleen and thymus are of especial interest. Their weights appear to be only slightly affected by body weight; the coefficients for actual weight are comparatively small but are slightly larger than those for relative weight so that the results agree with the superficial lymph nodes in one respect and with the deep lymph nodes in another. The closest analogy, however, is with the parathyroids which have coefficients that are almost identical with those of the thymus. This is of interest as we have additional evidence of a relation between these organs.

The endocrine glands show a variety of conditions. The suprarenals and the thyroid give results which differ chiefly with respect to the magnitude of the coefficients. The coefficients for the actual weight of the suprarenals are comparatively large while those for relative weight are very small giving a value for the direct relation which is considerably higher than that of any other organ in this group. The significant feature of the relation shown by these two organs is, however, the constancy of the weight per kilo of body weight within certain limits (Text-fig. 1).

The hypophysis and pineal gland show a relation to body weight

which is the reverse of that shown by the suprarenals and thyroid. In the case of the hypophysis, both sets of coefficients are large but the negative value is larger than the positive so that the reduction in the weight of the organ per kilo of body weight is more clearly defined than the increase in the actual weight of the organ. The pineal gland exhibits a similar tendency with an even greater discrepancy between positive and negative values. The brain and the superficial lymph nodes are the only other organs that show such a decided preponderance of the inverse over the direct relation between body and organ weight.

As has been pointed out, the parathyroids give results that are more nearly comparable to those of the thymus than to other endocrine glands so far as correlation coefficients are concerned. Both sets of coefficients are small and, while the curves for mean weights are decidedly irregular, neither the actual nor the relative weight of the organs appears to be materially affected by body weight. In fact, if we disregard the upper and lower ends of the curve, the results obtained show the nearest approximation to a neutral equilibrium that is given by any of the organs studied.

The significance of the points brought out by this discussion may be made clearer by tabulating the results for gross body weight in the following manner:

Organ	Coefficient		Organ	Coefficient		Organ	Coefficient	
	Actual	Relative		Actual	Relative		Actual	Relative
Heart.....	+0.755	−0.169	(Testicles)	+0.555	+0.101	Deep cervicals..	+0.246	−0.058
(Testicles).	+0.555	+0.101	Thyroid..	+0.248	−0.019	Mesenterics....	+0.307	−0.143
			Suprarenals...	+0.364	−0.066			
Gastrointestinal mass....	+0.456	−0.311	Parathyroids....	+0.221	−0.174	Spleen....	+0.186	−0.113
Kidneys...	+0.471	−0.443				Thymus...	+0.222	−0.160
Liver.....	+0.291	−0.301						
Brain.....	+0.451	−0.761	Hypophysis...	+0.343	−0.402	Popliteals..	+0.190	−0.271
			Pineal....	+0.193	−0.316	Axillaries..	+0.074	−0.321

This arrangement of organs takes into account structural and functional relationships as well as the correlation between body and organ weight. The organs studied are divided into three main groups; each of these groups contains three subdivisions, which differ with respect to the relative magnitude of the coefficients for actual and relative weights, arranged in the order of a diminishing direct or increasing inverse relation. The table may be read in any direction. In general, it will be seen that the magnitude of the coefficients for corresponding subdivisions, and hence the closeness of the relation between body and organ weight, diminishes from left to right; in like manner, the direct relation, which is strongest in the first subdivision of each group, diminishes and then changes to an inverse relation. The extreme conditions are represented by the upper left and the lower right divisions.

Tabulation of the results on the basis of the coefficients for net body weight, as has been pointed out above, merely accentuates the direct relation of the endocrine glands and the inverse relation of other organs with only a few minor changes in the actual arrangement of the organs in any given subdivision.

A number of interesting deductions may be drawn from this study. As has already been pointed out, the relations between body and organ weight are diverse. There are a number of organs that show a comparatively high and undoubtedly significant correlation with body weight but only a few that show a correlation of a very high order. In some cases it is the direct relation that is significant, in others, the indirect relation overshadows the direct and, in still other instances, the two are of about equal rank. There are only two clearly defined instances, however, of a direct relation between the actual weight of an organ and that of the body which is sufficiently close to maintain a constant ratio between body and organ weight. In most cases, the relations are such as to favor a diminishing weight per kilo of body weight. For animals weighing less than 2200 to 2300 gm., the weight of the organ exceeds the mean value for animals of all groups while beyond this point the weight becomes less than the mean (Text-fig. 1) so that, as a rule, the larger the animal the smaller the mass of organ tissue per unit of body weight that is available to perform a given function. The amount of the reduction in propor-

tion to body weight varies with different organs, but, as a matter of interest, it may be pointed out that, in several instances the correlation coefficients for actual weight give a rough approximation of the part of the organ that is supposed to be essential to the performance of its function, so far as such information is available, or that the coefficient is the reciprocal of the fractional part of the organ or tissue that may be removed without causing serious impairment of function.

Finally, attention should be called to the fact that the results reported above do not represent conditions that obtain in strictly normal rabbits of a given age and breed. They are reported with a realization that not only these but still other factors may have affected the values obtained. There is, however, substantial evidence that the relations found between body and organ weight have an important bearing on the problem of physical constitution and that the results have both an anatomic and a functional significance. In general, it appears that organs that are related anatomically or that may be supposed to perform analogous or related functions give results of a comparable nature, both with respect to the magnitude and the form of the relation shown.

SUMMARY.

Data from 645 normal rabbits were used as the basis of an investigation of the relation existing between body and organ weights. Actual and relative weights were correlated with both gross and net body weight.

The results obtained varied with different classes of organs but it was found that, in general, there was an agreement between the form and degree of the correlation shown and the structural and functional properties of the organs concerned.

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THE OCCURRENCE AND TREND OF SPONTANEOUS VARIATIONS IN ORGAN WEIGHTS OF NORMAL RABBITS.

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It is generally recognized that the weights of organs, in proportion to body weight, show wide variations in animals of a given species, that they may differ in the two sexes, and that the ratio of organ to body weight changes with age or development. It has also been found that the weights of some organs, notably the endocrine glands, may be affected by other factors such as dietary and climatic conditions and by sexual activity and that some of these conditions tend to produce periodic or even cyclic changes in weight. These conceptions have been derived chiefly from observations on small groups of animals, studied under various conditions, and, usually, with especial reference to a particular organ or to the influence of some particular condition, so that at the present time there is no clearly defined conception of what this series of variations represents in mature animals of a given sex from the standpoint of consecutive changes affecting the animal organism as a whole over long periods of time. This aspect of the subject of organ weight is, however, of the foremost importance in the study of almost any phase of the problem of physical constitution.

In previous papers (1) we have reported results of weight determinations on a large group of normal rabbits with especial reference to mean values, distribution frequencies, constants which, in general, define the variability of organ weights, the probable limits of variation, and the relations that obtain between the weight of the animal and the weight of organs. Thus far, we have dealt primarily with normal standards but we have emphasized the fact that standard values must be used with extreme caution due to the occurrence of

wide variations in weight, the time of occurrence and extent of which are unpredictable and for which the causes are still unknown.

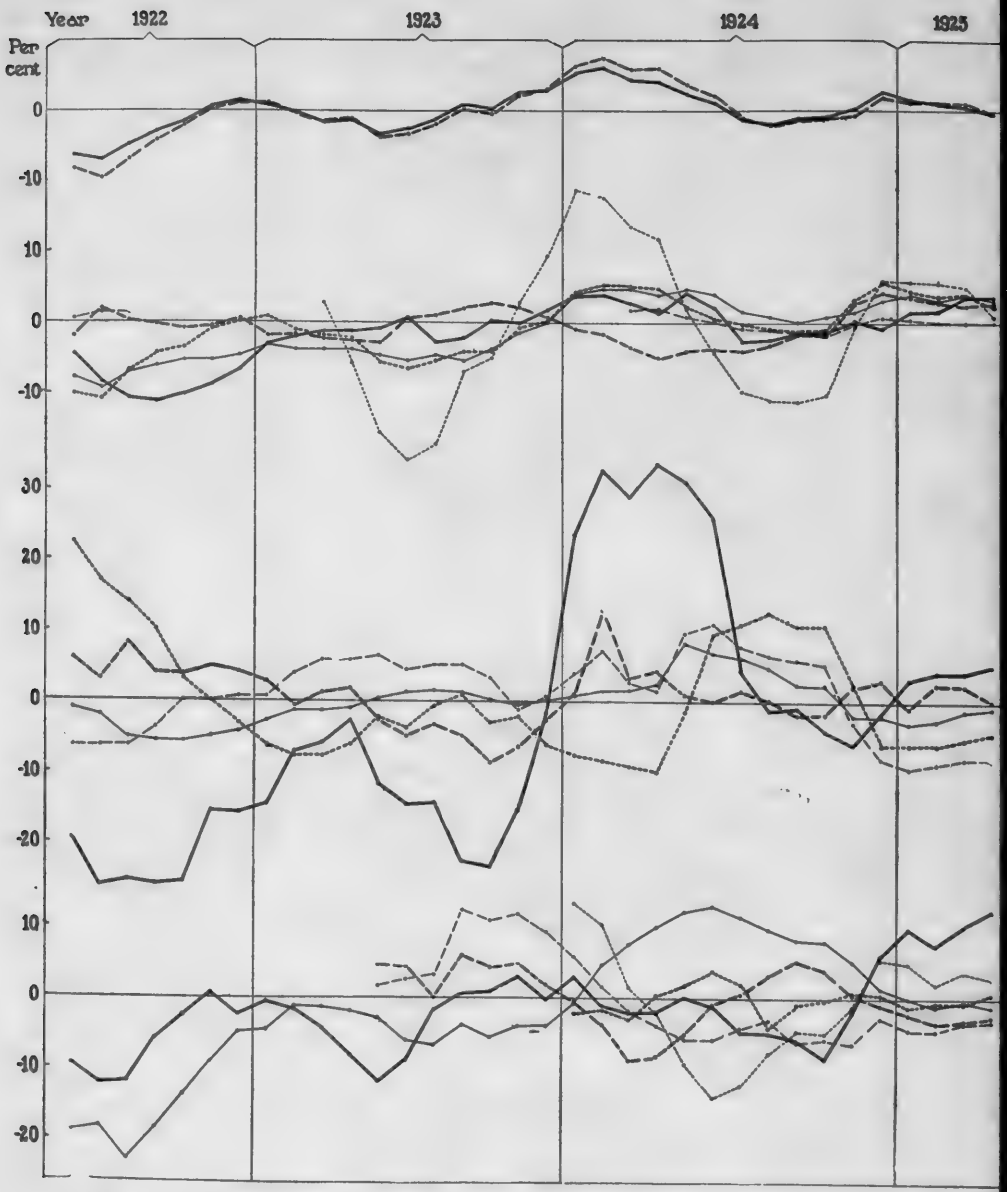
As a means of providing a background for the consideration of more detailed aspects of the problem of the occurrence of variations in organ weight, it seemed desirable to approach the subject with a view to determining first, whether any significant variations in weight of an orderly character could be demonstrated and second, the general course of the variations observed. The purpose of this paper is, therefore, to report the results obtained from a study of the general trend of consecutive variations in mean organ weights of normal male rabbits over a period of $3\frac{1}{2}$ years. The details of the changes observed from month to month and the cause of the variations in weight will not be considered at this time.

Methods and Material.

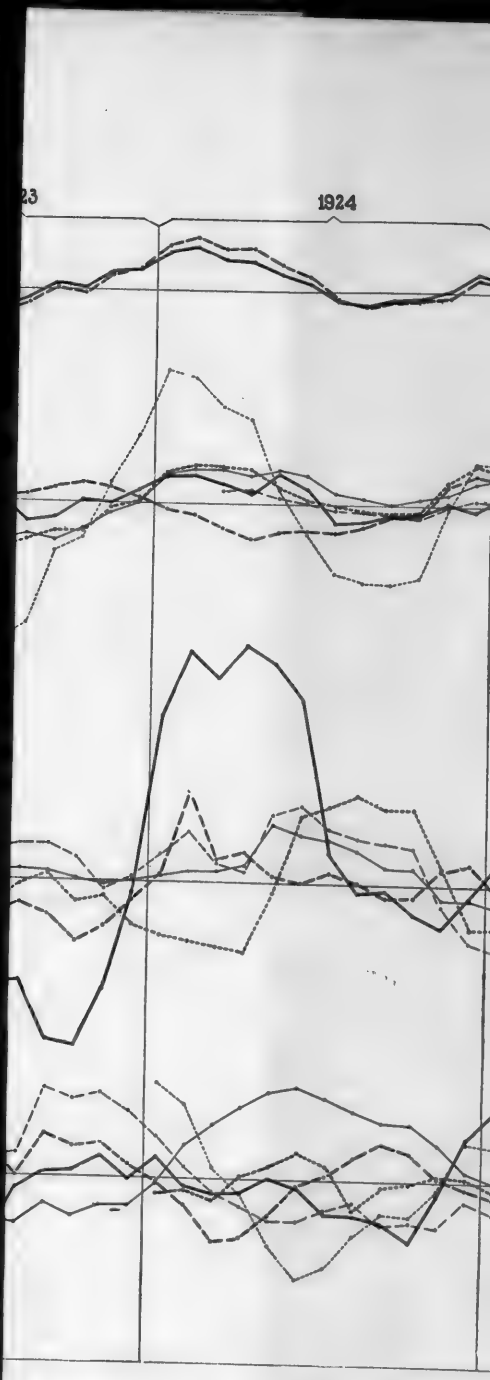
The material on which this report is based and the methods of investigation that were employed in collecting the data have been described in previous papers (2). We have used results of weight determinations from 645 male rabbits killed in small groups between January 1, 1922, and July 1, 1925. The animals of different groups were fairly comparable as to source, age, and breed; they were given a uniform diet throughout the period covered by the observations but it is not unlikely that the quality of the diet varied and there were some irregularities in the length of time different groups of animals were caged and held under observation before the final weight determinations were made.

The results are presented in the form of a table (Table I) and a series of text-figures (Text-figs. 1 and 2). The actual weights of organs and the weights per kilo of net body weight are recorded in Table I on the basis of the mean values of 39 monthly groups arranged in consecutive order.[1] From these data we have plotted a series of (partially) smoothed curves (Text-figs. 1 and 2) which show the trend of variations in weight from January, 1922, to June, 1925, inclusive, in the form of percentage deviations from the mean normal value. The method of smoothing that was employed is that of a moving weighted average using six monthly groups as the weighting unit according to the following formula:

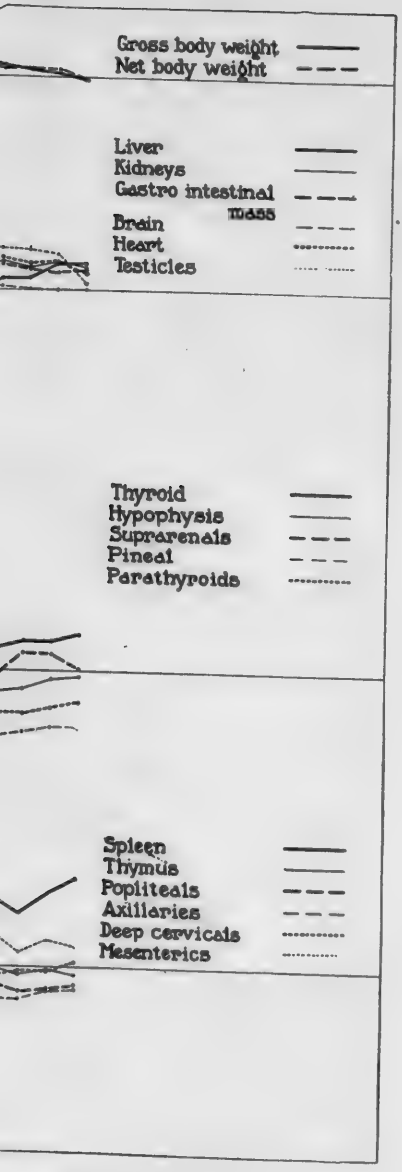
[1] During the first few months of this investigation, a number of animals that had been used for some minor experimental test was included in the series for normal weight determinations. Subsequently, the data from all of these animals were excluded. In this way all results for July, 1922, were eliminated and the number of animals in other groups was considerably reduced, apparently without materially affecting the results.



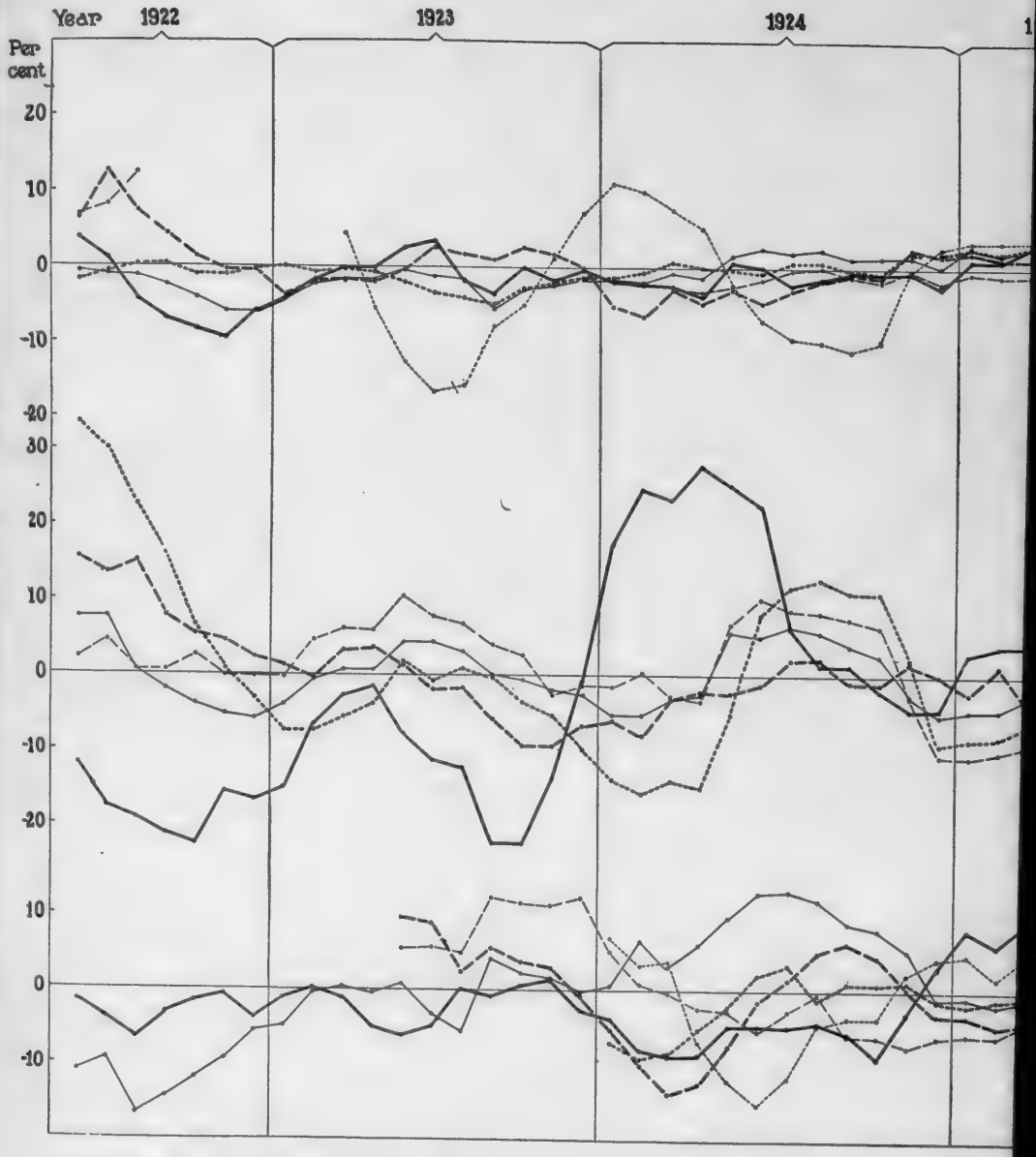
TEXT-FIG. 1. Variations in body and actual organ weight in terms of the percentage deviation



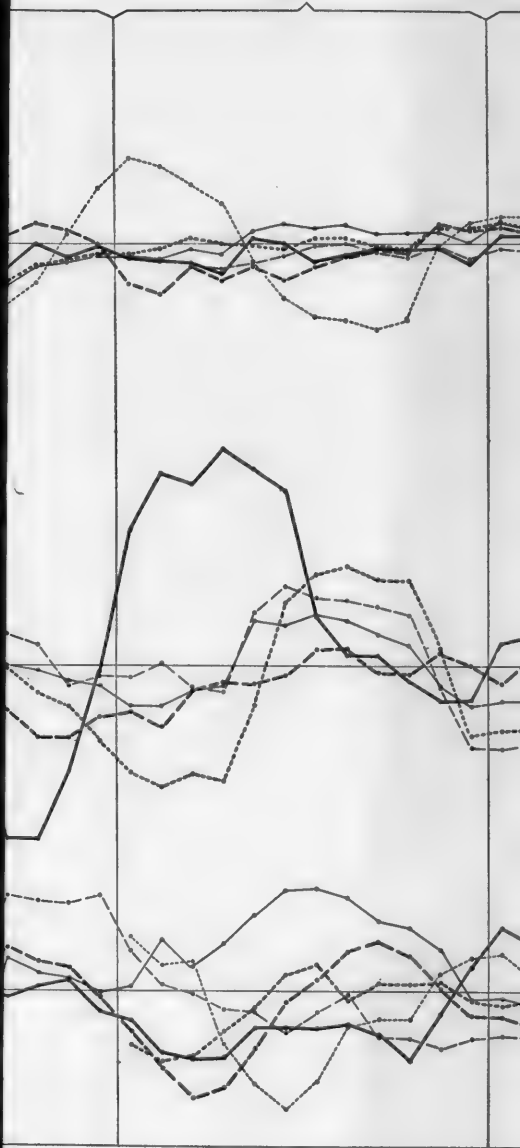
nd actual organ weight in terms of the percentage



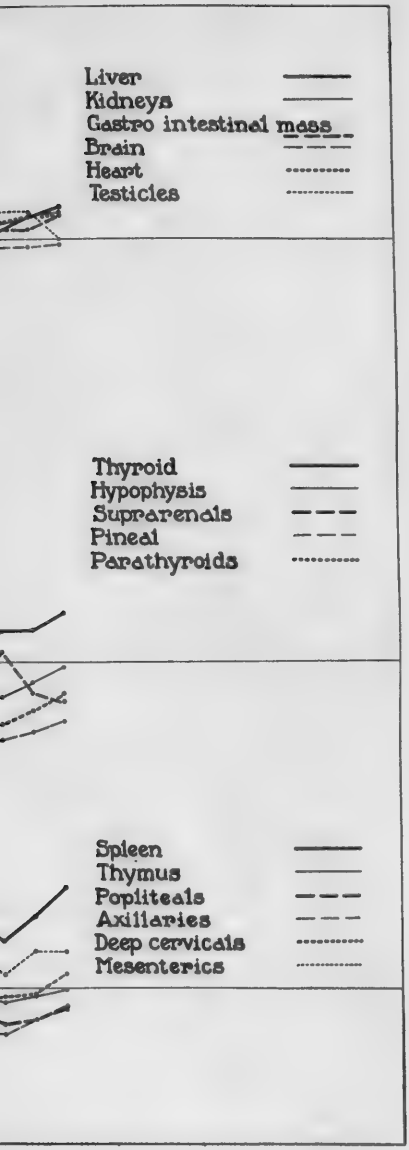
deviation from the mean value.



TEXT-FIG. 2. Variations in the weight of organs per kilo of net body weight in terms of the perc



of organs per kilo of net body weight in terms of the p



ercentage deviation from the mean.

$\frac{m_1 + m_2 + m_3 + m_4 + m_5 + m_6}{N}$ in which m represents the weighted total for a given month and N the total number of animals for the 6 months period. By this method, major variations are brought out clearly and at the same time some indication of short period variations is retained which we regard as desirable in order to avoid giving an erroneous impression of smooth or perfect continuity.

RESULTS.

The results obtained and the data on which they are based are presented in Text-figs. 1 and 2 and Table I.

DISCUSSION AND CONCLUSIONS.

The results recorded in Table I and Text-figs. 1 and 2 bring out a number of important points concerning the weights of given organs in normal animals, the relation of the weight of one organ to that of others, and the occurrence of variations in weights and relationships. A critical analysis of any of these points would require reference to data which, as yet, have not been presented, but we may refer briefly to a few conditions that are suggested by the smoothed curves in order to clarify certain features of the general tendency that is shown by these curves.

In the first place, there can be no question as to the occurrence of significant variations in the weights of many organs. During the period covered by these observations, it will be noted that all of the organs studied showed apparent variations in weight which assumed the general form of annual cycles. By reference to the tabulated values for mean weight (Table I), it will be seen, however, that the actual variations in weight from month to month were not as uniform as the curves would indicate and that the exact time of occurrence of maximum and minimum weights were in reality somewhat irregular. For example, it is generally stated that the thyroid of animals is largest during the first 4 months of the year, or during the winter, and the curves for thyroid weights would seem to support this view. The figures in Table I show, however, that this is not strictly correct. Exceptionally low values may be obtained at this period of the year and very high values may be obtained for months during which the thyroid is supposed to be small. Perhaps the most constant condition shown by the thyroid, in this case, is the occurrence of low and

TABLE I.

Mean Values for Actual and Relative Weights.

Month	No. of animals	Gross body weight	Net body weight	Gastrointestinal mass		Heart		Liver		Kidneys		Spleen	
				Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
				gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1922													
January	2	2263	1760	503	284	5.08	2.92	106.0	60.6	12.3	6.94	0.640	0.356
February	5	2000	1560	423	272	4.50	2.89	76.6	48.9	10.5	6.75	0.968	0.620
March	6	2408	1918	467	246	5.38	2.81	94.5	50.0	12.8	6.74	1.005	0.535
April	6	2050	1622	413	260	4.66	2.91	81.9	53.1	12.1	7.52	0.810	0.510
May	5	2075	1683	381	227	4.71	2.83	84.2	50.3	12.9	7.65	0.828	0.490
June	12	2050	1654	378	230	4.56	2.75	72.9	44.5	11.6	6.98	1.017	0.621
September	10	2090	1579	502	323	4.63	2.97	71.3	45.9	11.0	6.96	0.752	0.459
October	17	2229	1814	403	223	5.36	2.96	72.0	39.9	12.5	6.91	0.927	0.515
November	14	2464	2025	432	218	5.72	2.85	82.4	41.6	13.0	6.54	1.230	0.620
December	15	2290	1882	403	216	5.09	2.70	83.1	44.4	12.3	6.57	1.070	0.576
1923													
January	17	2415	1985	417	212	5.69	2.87	84.5	42.8	12.8	6.45	1.105	0.558
February	13	2187	1776	407	232	5.01	2.84	85.8	49.1	12.2	6.99	0.820	0.498
March	15	2088	1675	413	245	5.04	3.02	92.3	56.4	12.3	7.46	0.940	0.577
April	17	2097	1683	406	243	4.81	2.84	76.4	46.3	12.4	7.78	0.876	0.559
May	12	2290	1871	414	220	5.44	2.90	88.1	46.9	12.6	6.78	1.050	0.570
June	9	2347	1950	397	204	4.89	2.51	82.1	42.3	12.2	6.24	0.773	0.388
July	10	2193	1781	412	230	4.71	2.64	86.7	48.1	12.1	6.77	0.941	0.531
September	15	2303	1824	479	269	4.77	2.63	92.7	51.3	12.0	6.69	0.988	0.536
October	14	2234	1809	423	235	5.34	2.92	76.9	43.1	13.0	7.23	1.340	0.735
November	15	2300	1870	427	231	5.12	2.75	77.9	42.5	11.7	6.56	0.976	0.531
December	15	2238	1797	431	243	5.38	3.01	98.0	55.1	13.8	7.77	1.058	0.610

1924													
January	18	2475	2054	421	214	5.85	2.89	82.7	41.2	13.5	6.76	1.010	0.500
February	17	2307	1920	387	208	5.23	2.79	95.1	50.7	13.5	7.17	0.801	0.423
March	20	2638	2192	432	201	5.92	2.72	99.6	46.8	14.6	6.75	1.162	0.504
April	15	2345	1947	398	212	5.71	2.96	78.4	41.6	13.4	6.96	1.043	0.560
May	22	2160	1773	387	222	5.27	3.00	76.8	43.4	12.7	7.25	0.935	0.512
June	14	2177	1791	386	220	5.15	2.92	90.4	51.2	13.0	7.40	1.068	0.619
July	19	2253	1827	426	236	5.04	2.80	96.2	53.4	14.1	7.93	1.130	0.622
August	20	2135	1743	392	234	4.82	2.77	84.3	48.6	13.1	7.58	0.773	0.446
September	32	2305	1887	415	223	5.54	2.91	79.8	42.9	12.7	6.80	0.962	0.514
October	15	2227	1803	423	238	5.42	3.00	79.4	44.7	12.9	7.22	1.030	0.579
November	19	2282	1856	426	232	5.14	2.78	81.2	45.2	12.4	6.71	0.864	0.465
December	14	2210	1820	390	217	5.20	2.87	91.9	50.8	13.5	7.44	0.844	0.458
1925													
January	45	2332	1851	476	262	5.90	3.08	94.0	51.7	14.0	7.57	1.239	0.669
February	40	2422	1983	439	227	5.68	2.88	81.2	42.3	13.7	7.04	1.223	0.636
March	27	2169	1771	398	229	5.17	3.01	90.6	52.5	13.4	7.87	1.170	0.673
April	30	2246	1833	413	227	5.25	2.88	83.8	46.1	12.5	6.90	0.943	0.523
May	20	2199	1792	407	227	5.21	2.91	96.6	53.2	13.2	7.37	1.119	0.636
June	14	1959	1545	404	263	4.14	2.67	91.4	58.5	12.2	7.90	1.113	0.720

TABLE I—*Continued.*

Month	No. of animals	Thymus		Brain		Testicles		Thyroid		Parathyroids		Hypophysis	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
1922		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
January	2	1.802	1.047	9.63	5.0	6.63	3.75	0.2350	0.1318	0.0200	0.0117	0.0260	0.0149
February	5	1.687	1.093	8.54	5.49	5.77	3.69	0.1356	0.0871	0.0134	0.0086	0.0302	0.0195
March	6	1.983	1.055	9.32	4.89	6.15	3.18	0.2125	0.1115	0.0173	0.0091	0.0292	0.0154
April	6	1.906	1.245	9.47	5.96	6.60	4.05	0.1737	0.1088	0.0168	0.0106	0.0280	0.0176
May	5	2.181	1.288	9.31	5.59	4.50	2.64	0.1820	0.1094	0.0134	0.0079	0.0256	0.0152
June	12	1.745	1.059	9.49	5.74	4.04	2.47	0.1973	0.1208	0.0159	0.0097	0.0275	0.0167
September	10	1.915	1.199	8.60	6.10			0.1306	0.0836	0.0137	0.0088	0.0261	0.0165
October	17	1.480	0.824					0.1672	0.0929	0.0132	0.0073	0.0254	0.0141
November	14	2.328	1.190					0.1840	0.0936	0.0137	0.0068	0.0271	0.0135
December	15	2.414	1.299			6.29	3.52	0.1779	0.0953	0.0111	0.0059	0.0271	0.0146
1923													
January	17	2.570	1.324			5.49	2.68	0.2927	0.1465	0.0112	0.0056	0.0274	0.0140
February	13	2.423	1.376			4.69	2.62	0.1936	0.1103	0.0132	0.0078	0.0286	0.0163
March	15	2.060	1.252			4.61	2.73	0.1681	0.1030	0.0105	0.0063	0.0286	0.0173
April	17	1.887	1.132			3.15	1.93	0.2602	0.1504	0.0121	0.0073	0.0276	0.0169
May	12	2.313	1.256			4.66	2.47	0.1992	0.1071	0.0143	0.0078	0.0273	0.0147
June	9	2.383	1.216			3.65	1.75	0.2174	0.1147	0.0127	0.0065	0.0272	0.0139
July	10	2.638	1.481			2.68	1.53	0.1816	0.1026	0.0141	0.0078	0.0301	0.0169
September	15	2.006	1.099			3.68	2.03	0.1599	0.0874	0.0122	0.0068	0.0300	0.0165
October	14	1.980	1.094			5.23	2.88	0.1683	0.0951	0.0123	0.0069	0.0291	0.0163
November	15	2.172	1.736			5.46	2.92	0.1733	0.0924	0.0133	0.0070	0.0269	0.0145
December	15	2.088	1.150	9.11	5.37	5.06	2.80	0.1856	0.1047	0.0114	0.0064	0.0261	0.0147

1924													
January	18	2.449	1.208	9.30	4.73	5.83	2.85	0.3336	0.1601	0.0128	0.0061	0.0268	0.0132
February	17	2.463	1.275	9.02	4.77	5.30	2.77	0.3647	0.1954	0.0107	0.0056	0.0301	0.0160
March	20	2.417	1.166	9.58	4.45	6.12	2.79	0.4666	0.2105	0.0115	0.0053	0.0304	0.0141
April	15	2.833	1.503	9.62	5.07	4.93	2.55	0.3038	0.1600	0.0114	0.0060	0.0301	0.0158
May	22	2.580	1.493	9.32	5.35	4.57	2.59	0.1852	0.1038	0.0124	0.0072	0.0280	0.0161
June	14	2.520	1.425	9.17	5.23	4.42	2.46	0.2611	0.1452	0.0104	0.0059	0.0268	0.0154
July	19	2.716	1.480	8.78	4.92	3.23	1.72	0.2555	0.1421	0.0193	0.0107	0.0372	0.0209
August	20	2.581	1.513	8.70	5.06	3.59	2.05	0.2883	0.1704	0.0185	0.0108	0.0274	0.0157
September	32	2.360	1.270	9.10	4.87	4.51	2.40	0.2092	0.1121	0.0135	0.0072	0.0297	0.0158
October	15	2.480	1.391	9.41	5.29	4.49	2.49	0.1983	0.1099	0.0124	0.0067	0.0264	0.0148
November	19	2.375	1.268	9.11	4.98	4.55	2.43	0.1826	0.1005	0.0114	0.0063	0.0247	0.0136
December	14	2.477	1.341	8.97	4.97	4.87	2.69	0.1971	0.1080	0.0106	0.0057	0.0266	0.0146
1925													
January	45	2.377	1.298	9.39	5.16	5.23	2.83	0.2226	0.1214	0.0126	0.0069	0.0279	0.0152
February	40	2.144	1.091	9.26	4.77	5.40	2.62	0.2938	0.1457	0.0115	0.0057	0.0278	0.0142
March	27	2.170	1.286	8.93	5.25	4.34	2.45	0.2728	0.1622	0.0131	0.0075	0.0280	0.0164
April	30	2.251	1.243	9.18	5.05	4.69	2.57	0.2340	0.1277	0.0123	0.0068	0.0276	0.0151
May	20	2.490	1.374	8.98	5.05	4.29	2.42	0.1830	0.1024	0.0132	0.0074	0.0278	0.0157
June	14	2.275	1.464	9.01	5.89	2.47	1.59	0.2275	0.1450	0.0119	0.0078	0.0286	0.0186

TABLE I—*Concluded.*

Month	No. of animals	Suprarenals		Pineal		Popliteal lymph nodes		Axillary lymph nodes		Deep cervical lymph nodes		Mesenteric lymph nodes	
		Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative	Actual	Relative
1922		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
January	2	0.3885	0.2238	0.0120	0.0062							3.65	2.03
February	5	0.3228	0.2084	0.0163	0.0107								
March	6	0.5193	0.2769	0.0175	0.0092								
April	6	0.4165	0.2566	0.0127	0.0080								
May	5	0.3556	0.2060	0.0136	0.0081								
June	12	0.4032	0.2449	0.0150	0.0093								
September	10	0.3545	0.2183	0.0142	0.0091								
October	17	0.4366	0.2382	0.0153	0.0085								
November	14	0.3868	0.1874	0.0179	0.0089								
December	15	0.4020	0.2134	0.0173	0.0094								
1923													
January	17	0.4069	0.2057	0.0148	0.0075	0.2700	0.1360	0.1650	0.0830				
February	13	0.3816	0.2136	0.0158	0.0091	0.2715	0.1531	0.1790	0.1022				
March	15	0.3394	0.2027	0.0148	0.0089	0.3410	0.2065	0.2041	0.1229				
April	17	0.3667	0.2194	0.0182	0.0109	0.2440	0.1483	0.1487	0.0897				
May	12	0.4392	0.2415	0.0185	0.0099	0.2533	0.1382	0.1929	0.1043				
June	9	0.4283	0.2209	0.0177	0.0093	0.2166	0.1109	0.1611	0.0835				
July	10	0.2922	0.1589	0.0163	0.0093	0.2552	0.1459	0.1437	0.0818				
September	15	0.3333	0.1795	0.0144	0.0080	0.2683	0.1472	0.1863	0.1022				
October	14	0.3740	0.2056	0.0152	0.0085	0.2850	0.1599	0.2124	0.1206	0.2092	0.1198	3.48	1.95
November	15	0.3357	0.1784	0.0183	0.0099	0.3177	0.1714	0.2230	0.1229	0.1510	0.0789	3.81	1.95
December	15	0.3507	0.1943	0.0165	0.0091	0.2355	0.1272	0.1787	0.1013	0.1105	0.0666	4.03	2.23

1924													
January	18	0.4277	0.2051	0.0139	0.0069	0.2492	0.1228	0.1819	0.0924	0.1353	0.0671	4.48	2.24
February	17	0.3996	0.2008	0.0175	0.0095	0.2173	0.1158	0.1394	0.0965	0.1206	0.0623	4.08	2.14
March	20	0.4128	0.1904	0.0174	0.0081	0.2368	0.1099	0.1573	0.0749	0.1894	0.0834	3.62	1.68
April	15	0.3653	0.1873	0.0182	0.0095	0.2255	0.1191	0.1619	0.0853	0.2100	0.1050	2.80	1.46
May	22	0.4000	0.2309	0.0145	0.0083	0.2325	0.1316	0.1782	0.1084	0.1414	0.0812	2.35	1.36
June	14	0.3759	0.2115	0.0151	0.0087	0.2484	0.1424	0.1529	0.0873	0.1493	0.0863	3.16	1.79
July	19	0.3545	0.2018	0.0214	0.0119	0.2927	0.1628	0.1632	0.0909	0.1482	0.0826	2.97	1.65
August	20	0.3779	0.2134	0.0186	0.0111	0.2835	0.1635	0.1422	0.0813	0.1443	0.0828	3.00	1.72
September	32	0.4236	0.2233	0.0154	0.0082	0.2547	0.1374	0.1682	0.0892	0.1680	0.0894	3.61	1.94
October	15	0.3263	0.1792	0.0166	0.0093	0.2823	0.1583	0.1787	0.0991	0.1323	0.0729	4.09	2.30
November	19	0.3521	0.1934	0.0139	0.0076	0.2591	0.1401	0.1484	0.0798	0.1732	0.0925	2.92	1.56
December	14	0.3789	0.2074	0.0144	0.0080	0.2214	0.1216	0.1568	0.0862	0.1546	0.0853	3.12	1.72
1925													
January	45	0.4143	0.2231	0.0144	0.0078	0.2486	0.1350	0.1580	0.0855	0.1585	0.0856	3.82	2.10
February	40	0.3983	0.2003	0.0138	0.0070	0.2550	0.1313	0.1763	0.0889	0.1492	0.0766	3.88	1.99
March	27	0.3399	0.1945	0.0144	0.0082	0.2352	0.1365	0.1594	0.0925	0.1546	0.0875	3.46	2.00
April	30	0.4283	0.2348	0.0159	0.0088	0.2498	0.1365	0.1693	0.0937	0.1497	0.0815	3.27	1.79
May	20	0.3500	0.1962	0.0150	0.0085	0.2665	0.1475	0.1663	0.0930	0.1690	0.0950	3.54	1.98
June	14	0.2736	0.1794	0.0142	0.0092	0.2366	0.1526	0.1605	0.1017	0.1782	0.1130	2.76	1.80

comparatively uniform values during the months of September, October, and November, and even in December, rather than the occurrence of high values from January or December to April inclusive. Moreover, it will be seen that the thyroid showed a late spring or early summer increase in weight with as much regularity as it did the more generally recognized winter increase which, after all, may be comparatively slight and of irregular occurrence and duration, with intervening periods during which the weight of the organ is small.

These conditions are mentioned in order to make it clear that, while there is an evident tendency to the occurrence of cyclic variations in the weights of many organs with distinct seasonal relations, these changes do not occur with perfect regularity or in perfect conformity with the (astronomical) progression of the seasons. At times, the departure from the usual course is very decided.

A second point to be noted is the variation in the magnitude of the change from year to year. This is again illustrated best by the thyroid which shows that, in addition to the annual cycle, there was a progressive increase in the magnitude of the values obtained over a period of at least 3 years. Scattered observations made during the latter part of 1921, which are not included in the results recorded above, indicate that the maximum weight of the thyroid for the winter of 1921–22 was approximately the same as that given for the winter of 1922–23; it may have been a little higher or a little lower. During the winter of 1924–25, the weight was lower than for the preceding winter but it was still high (see Table I) and, while systematic observations were discontinued in June, 1925, sufficient data have been accumulated to make it certain that the weight of the thyroid increased during 1925–26 to a much higher level than any hitherto recorded by us. This applies to rabbits raised in our own laboratories as well as to those obtained from the usual outside sources which shows a widespread prevalence of a condition that might even be termed a goitrous enlargement.

If all of these observations are taken into account, the results obtained suggest the occurrence of a second cycle of weight variation extending over a period of years. The indications are that our observations may have been started at or near a period of minimum

thyroid weight (a period of maximum weight for some organs) or at the turning point of a decreasing phase of a cycle and that most of our observations fell within the limits of an increasing phase, the maximum of which we are now approaching or have reached during the past few months.

A third feature of the results to which attention may be directed is the suggested relation of the change in the weight of one organ to that of other organs. This is also shown best by the relation of the curves for the thyroid to those for other members of the endocrine system but it will be seen that still other organs show something of the same relation to the thyroid. In general, it may be said that the weight curve for the thyroid stands alone while those for the other endocrines tend to group themselves together and to move in a direction opposite to that of the thyroid with a definite lag in the movements. Still, these relations are not entirely constant. At times, all of the curves are more or less parallel and move in the same direction or they display irregularities in their movements. There is, however, a definite suggestion of a coordination of movements and apparently the manner in which this coordination is effected is influenced to some extent by the actual as well as the relative positions of the organs with respect to the mean normal or zero line. Thus, during 1922 and the early part of 1923 there was an apparent tendency for the weights of all organs to converge toward the mean normal. For a time thereafter, weights were comparatively stable while movements were more or less parallel and in the same direction. But, with the subsequent marked increase in the weight of the thyroid, the curves for other organs continued their downward course, initiating a series of reverse movements which were very decided and clearly defined during the greater part of 1924. This series of changes was followed in time by a second period of comparative stability and parallel coordination of movements which differed in some respects from that of 1923. This apparent variation in the coordination of weights at different levels with respect to the mean normal illustrates the difficulty of attempting to deduce a general rule governing the relation of one organ to another.

Finally, attention may be called to the great difference in the magnitude of the variation in weight shown by different organs.

The changes exhibited by the heart, brain, kidneys, liver, and gastrointestinal mass are of an entirely different order from those of the endocrine glands, the testicles, and lymphoid tissues. This raises the question of the significance of the variations shown and, while it is not our intention to enter into a general discussion of this aspect of the subject at the present time, it seems desirable to refer briefly to the possible influence on organ weight of certain factors that are inherent in the material, namely, the age and weight of animals.

The curves for gross and net body weight in Text-fig. 1 show periodic variations similar to those of the curves for organ weight. The variations in these curves represent not only differences in body weight but differences in age as well and are due to unavoidable conditions of animal supply. For example, during the winter months the supply of fully mature animals is more abundant than at any other time; as the supply of such animals becomes depleted, it is necessary to use a larger proportion of younger and smaller animals during the spring, summer, and early fall. These conditions are repeated year after year; they are reflected in the body weight curves and undoubtedly do have an effect on the curves for organ weight.

The factors of age and weight are so intimately bound up with each other that we cannot attempt to make any distinctions at this time but some indication of the influence that might be expected from this combination of factors may be obtained from the correlation coefficients for organ and body weight (3) which were calculated without distinction as to age so that they are directly applicable to the present instance. On this basis, one would expect that the body weight factor would affect the weights of the heart, testicles, kidneys, gastrointestinal mass, and brain more than other organs as their correlation coefficients are larger, and a careful comparison of the curves will show that the curves for actual and relative weights of these organs do conform most closely with those for body weight. Moreover, the degree of conformity is roughly proportional to the magnitude of the correlation coefficients. The gastrointestinal mass forms an exception and the extent of the variation shown by the testicles is out of proportion to that of other organs in this group.

It is evident, therefore, that, in the case of the organs mentioned, the body weight factor is of considerable importance and may indeed

account for the greater part of the variation shown. The liver appears to be less affected by this factor and a comparison of the curves for the endocrine glands and lymphoid tissues with those for body weight shows that the magnitude of the values and the movements of the curves are virtually independent of those for body weight. Still, it must not be assumed that the body weight factor had no influence on the results obtained for these organs as some of them show a fairly close correlation with body weight. On the whole, the results are in agreement with what might be expected on the basis of the correlation coefficients and it seems that, in the case of these organs, the influence of body weight was of subsidiary importance. There is some evidence, however, that, at times, the influence of this factor may have been such as to alter or mask the effects of other conditions and this possibility should not be overlooked.

It would appear, therefore, that the results recorded above give a composite picture of variations in weight due largely to external causes. At the present time, we are not concerned with point to point variations or with the exact magnitude of the change in weight shown but with the general course of events and the occurrence of changes that are so clearly defined and of such proportions that their significance cannot be questioned. The course of events represented by this series of observations may not agree in all respects with what might have been found had it been possible to follow the weights of organs in a given group of animals over the same period of time, but the manner in which the investigation was carried out and the nature of the results obtained are sufficient to warrant the conclusion that the picture presented gives a fair conception of changes in weight and weight relationships that actually occurred at this particular time.

If we consider these results from the point of view of normal standards, it is at once apparent that no fixed standard of weight or relation can be established. Conditions that prevail at one time differ greatly from those found at another and thus far we have no acceptable basis for making discriminations as to the normality of the findings for one period as compared with another. It seems that the animal organism is subject to the influence of extraneous conditions which determine the relations that obtain and the changes in

the weights of organs that must occur from time to time in order to maintain the health of the animal under changing conditions of life. Hence, weights and relations that would be regarded as normal for one period might be decidedly abnormal for another. Recognition of the occurrence of variations in weight as an essential attribute of organs and a knowledge of the extent to which the weights of various organs may be affected by environmental conditions is of fundamental importance in the study of problems of physical constitution from either an anatomic or functional point of view and is deserving of especial emphasis as the tendency at the present time is to stress standardization and conformity to rule rather than the occurrence of variations.

SUMMARY.

Records of organ weights of normal rabbits covering a period of $3\frac{1}{2}$ years were analyzed with a view to determining first, whether any significant variations of an orderly character could be demonstrated and, second, the general trend of the variations that occurred.

It was found that many organs showed decided variations in weight which assumed the form of definite annual cycles. In addition, there was some evidence of a second cycle covering a period of years. These changes were most marked in the case of the endocrine glands (including the testicles), the lymphoid organs, and the liver.

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STUDIES IN AGGLUTINATION.

III. On the Mechanism of the Agglutination of Bacteria by Specific Agglutinating Serum.

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INTRODUCTION.

The first hypotheses concerning the laws governing immunological reactions were characterized by considerable complexity. The effort to describe these phenomena in chemical-physical terms has led to much clarification. In the reaction of bacterial agglutination, in particular, this type of approach has been most productive, and the earlier hypothetical explanations of the reaction have been gradually replaced by conceptions which conform with general chemical-physical laws.

Very early in the study of the phenomenon of specific bacterial agglutination, Bordet (1) made the observation that when bacteria are treated with homologous agglutinin, no flocculation is possible until electrolytes have been added. He thus conceived of specific agglutination as occurring in two phases, *first*, sensitization of bacteria by agglutinating serum (union of antibody and antigen) and *second*, flocculation of the agglutinin-bacteria complex by the electrolytes of the suspending fluid. He considered this flocculation analogous to the precipitation of colloidal suspensions by salts. That this two-phase conception is essentially correct has been abundantly confirmed.

First Phase.—Little is known of the reason for the selectivity of the interaction between agglutinin and homologous organism, *i.e.*, specific sensitization. The explanation will probably be forthcoming only when the exact chemical nature of the two reacting substances is determined.

The general character of agglutinin is known. It has been shown to be closely associated with the serum globulin, as it is precipitated out of the serum in the globulin fraction, and remains with the globulin (either euglobulin, pseudoglobulin, or both (2)) after purification of the protein. Also, the isoelectric point of the

agglutinin has been shown to be identical with that of globulin, pH 5.2–5.4 (3, 4). This has been disputed recently (5), but our own experiments support this conclusion. Agglutinin is not part of the serum albumin (2) and is not removed by lipoid extraction of the serum (6). That agglutinins are globulins is still unsettled; but that the agglutinin-globulin complex is the essential reacting substance seems clear.

Very important advances have been made recently by Avery, Heidelberger, and their coworkers (7) and Zinsser, Mueller, *et al.* (8), in the direction of ascertaining the chemical constitution of the portions of the bacterial cell which take part in specific interactions with antibody. The first group of workers have isolated from the three fixed types of pneumococcus and from strains of *B. friedlænderi*, certain chemically distinct substances which they have shown to be polysaccharides, which react specifically with homologous antisera. These authors believe that these type-specific substances are to be found in the ectoplasmic layers of the cell and that, in view of the fact that the immune reactions are presumably cell surface phenomena, the presence in the periphery of the organisms of these substances determines the specificity and the readiness of response of the reaction. These workers have also isolated less clearly defined substances (nucleoproteins) which they have shown to be specific species-reacting substances. The second named investigators have shown that certain non-protein residue substances (carbohydrate gums) may be extracted from many types of bacteria and from yeasts, and that they bear a definite relation to the specific character of the organisms in that they react specifically with homologous immune sera.

To explain the mechanism of the union between antibody and organism, two main hypotheses have been suggested. The first, that the combination of the two is due to their possessing opposite electrical charges, seems to have been disposed of finally by Northrop and De Kruif (9) who have shown that union may occur when both elements are similarly charged.

The second explanation assumes that the antibody forms a film at the surface of the organisms and is held by ordinary forces of valence. A counterpart of this is found in the observation of Loeb (10) that collodion particles treated with proteins acquire a firmly fixed film of protein at their surfaces; a corollary, of much importance, in his work, is the observation that such film formation causes the particles to assume certain characteristics, notably the cataphoretic behaviour, of the proteins used.

In favour of the second hypothesis may be cited the following. Coulter (11) has shown that the optimum pH for agglutination of red blood cells is 4.75, but that when they are sensitized the optimum shifts to pH 5.3, *i.e.*, the isoelectric point of serum globulin. That is, in respect of their acid agglutination point, the red cells now behave like particles of euglobulin. Similar findings have been reported by Northrop and De Kruif (12) in experiments with bacteria and normal and immune sera, egg albumin and globin, and by Eggerth and Bellows (13) with bacteria and various proteins. That this assumption is correct seems to be established by certain of our experimental results that appear below.

Second Phase.—Much more is known of this aspect of the problem. Bechold (14), Neisser and Friedemann (15), and Arkwright (16), in support of Bordet, have emphasized the similarity between bacterial and colloidal suspensions in demonstrating the fact that bacteria possess an electrical charge, as evidenced by migration in an electrical field.[1] The two first named authors have carried the analogy further by demonstrating the similarity between the salt precipitation of kaolin and mastic, and of sensitized bacteria. They also have stated that sensitized bacteria lose their charge and have suggested that neutralization of charge is responsible for agglutination, recalling the observation of Hardy that suspensions of denatured protein (egg white) coagulate at the point at which they are devoid of charge; that this last assumption is partially incorrect appears later.

Tulloch (17) observed that, in respect of their behaviour toward electrolytes, unsensitized bacteria resemble fresh egg white and sensitized organisms resemble denatured egg white, *i.e.*, the former are precipitated by strong concentrations of salt only, while the latter flocculate readily in low salt, *e.g.*, physiological salt solution. He concludes that the process of sensitization is akin to denaturation of bacteria.

Buchanan (20), in an exhaustive review of the subject of agglutination, suggests that the like charges on bacteria act as forces of repulsion to keep the suspensions dispersed and that the force of surface tension acts as the attracting agency to cause agglutination. He suggests that agglutination occurs whenever factors are introduced which make the repelling force relatively less than the pull of surface tension.

The most valuable contribution to our knowledge of the chemical-physical laws governing bacterial agglutination is found in the recent work of Northrop and De Kruif (9, 12, 21). Their findings are so important, and so satisfactorily correlate the observations of previous investigators, that those conclusions bearing specifically upon the present consideration may well be reviewed at considerable length.

These authors accept the hypothesis that there are two factors making for repulsion, or for adhesion (and subsequent clumping) of bacterial particles; the first being the electrical charge on the organisms, and the second, a force, still insufficiently defined, to which they have assigned the name "cohesive force." They assume that, whenever the latter force is relatively higher than the former, agglutination results. However, they maintain that no satisfactory testing of these hypotheses is possible without measurement of both forces; accordingly they have devised a technique for measuring cohesive force. The method, as they indicate, is not one of absolute precision, but the values obtained are regularly reproducible; and in our hands (23) the method has given perfectly satisfactory results. Bacterial charge was measured from the rate of migration in a cataphoretic cell.

[1] For theoretical discussions of the origin of this charge see the work of Loeb (10, 18), Northrop and De Kruif (21), and Winslow, Falk, and Caulfield (19).

Their conclusions may be summarized briefly as follows: (1) Agglutination is to be considered in terms of two antagonistic forces: a *repelling force*, due to like electrical charge, which tends to keep the bacteria apart, and "*cohesive force*," which makes for adhesion. In any bacterial suspension, all factors that make the repelling force relatively greater than the cohesive force make for stability; and conversely, all factors that reduce the repelling force or otherwise make the cohesive force relatively greater, lead to flocculation. (2) In the case of unsensitized bacteria, electrolytes in lower concentrations, < 0.01 N, affect primarily the potential, and in higher concentrations, > 0.01–0.1 N, affect primarily the cohesive force. (3) As long as cohesive force is unaffected, agglutination occurs whenever the charge is reduced by electrolytes to a point below a critical level of about 15 millivolts;[2] that is, the unaffected cohesive force now becomes relatively greater than the force of repulsion. (4) Salt in high concentration depresses the cohesive force of unsensitized bacteria so that no agglutination occurs even though there may be no measurable charge; *i.e.*, the cohesive force is now so small that it is always less than the repelling force. (5) When bacteria are treated with immune sera, their cohesive force is in some manner protected from this depressing effect of strong salt (*e.g.*, physiological salt solution, etc.) and agglutination is determined solely by the charge; that is, whenever the potential of the sensitized bacteria is reduced by electrolyte to a point below 15 millivolts, the suspension agglutinates. This explains the observation of Bordet, confirmed by Northrop and De Kruif in the course of the work being cited, that electrolytes are essential for specific agglutination. That is to say, the salt, routinely used in the ordinary reactions, reduces the charge on the bacteria so that this charge comes to lie in the 15 millivolt agglutination zone, and, the cohesive force of the sensitized bacteria, being insusceptible to the depressing effect of the electrolyte, is now relatively greater than the repelling force and flocculation occurs.[3] (6) Their results refute the idea that combination of antibody and organism is caused by difference of sign of the charges carried by the two substances; but are in agreement with the assumption that the agglutinin forms a film on the surface of the organism.

In the course of studies concerned with the importance of environmental factors in agglutination (23), we have been much interested in the nature of the changes in electrical charge that occur when bacteria are treated with agglutinative sera. As a result of observations upon these changes, much light has been shed upon the general nature

[2] Loeb (22) has shown that a similar critical potential agglutinating zone exists for many varieties of suspended particles, *cf.*, native and denatured proteins, collodion, graphite, etc.

[3] That this may not be the only part played by electrolytes is indicated, however, in experiments still under investigation, with organisms having a low primary charge, less than 15 millivolts.

of the reaction, and we now feel that we have sufficient data upon which to found a satisfactory hypothesis for the mechanism of the agglutination of bacteria by specific serum.

Methods.

Pseudoglobulin was obtained from human ascitic fluid as follows: the globulin was thrown down with 50 per cent $(NH_4)_2 SO_4$ and the precipitate put into membranes and dialyzed against running tap water 48 hours. To this, $(NH_4)_2 SO_4$ to make 25 per cent, was added to remove the euglobulin—to the filtrate enough salt was added to make 50 per cent and the pseudoglobulin precipitate was redialyzed against tap and distilled water at pH 5.4. The precipitate in this was discarded. The resulting solution of pseudoglobulin was 4.5 per cent.

All other methods used, unless otherwise indicated, are similar to those described in the preceding paper (24).

EXPERIMENTAL RESULTS.

The first step in specific bacterial agglutination is selective interaction between agglutinin and organism. As will be recalled, the evidence thus far obtained favours the assumption that this union consists of a coating of the organism by agglutinin. Proof of the validity of this assumption is essential to the hypothesis for the mechanism of the reaction to be outlined below. The following experiments seem to establish the correctness of this conception of sensitization.

In a preceding paper (24), it has been shown that in the cases of Type I pneumococcus and *B. paratyphosus* A, their agglutinative sera possess a specific charge-reducing effect which is quantitatively related to the titer of the serum. It has been shown that this effect disappears following absorption of agglutinin, and that it is not present in highly protective sera that have no agglutinative properties.[4]

Fig. 1 illustrates this effect in the case of the Type I pneumococcus serum. It will be seen from this figure that both normal and immune sera depress the charge on the bacteria as their concentration is increased, but it will be observed that the normal serum effect is only slight while the reducing effect of the specific serum is striking.

[4] We have since observed also that the protective antibody of Huntoon (25), which is practically non-agglutinative, does not show this specific effect.

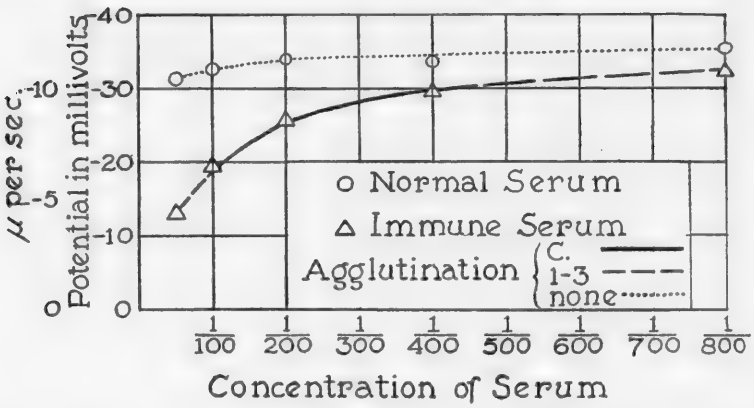

FIG. 1. Effect of normal serum and Type I pneumococcus serum on potential and agglutination of Type I pneumococcus. Experiment in M/200 glycocoll-phosphate-acetate buffer, pH 7.0. In this and subsequent figures complete agglutination is shown by a solid line, incomplete by a broken line, and absent agglutination by a dotted line. This figure is kindly reprinted from the previous paper (24).

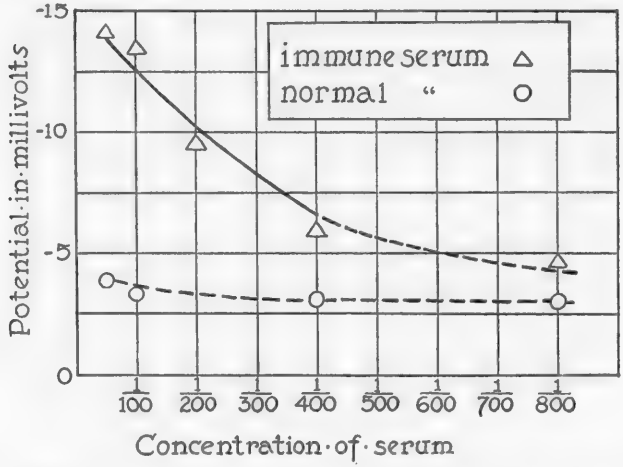

FIG. 2. Effect of normal serum and specific agglutinative serum upon *B. dysenteriæ*, Shiga strain. There was no agglutination by normal serum. Experiment in M/200 glycocoll-phosphate-acetate buffer, pH 7.0.

We have since extended observations of this phenomenon to several other bacteria and their agglutinating sera. The organisms studied now include, Type I pneumococcus, meningococcus, staphylococcus, *Streptococcus scarlatinæ*, a diplococcus variant of a hemolytic streptococcus, *B. coli*, *B. typhosus*, *B. paratyphosus* A, *B. tuberculosis*, and three strains of the dysentery bacillus, Flexner, Shiga, and Mt. Desert.

Confirmation of the fact that agglutinative sera possess this charge-reducing effect was obtained in all cases studied except the three dysentery strains. In the case of these organisms, an unexpected result was secured when it was found that their specific sera in high concentration not only do not reduce the charge, but raise it. Fig. 2 illustrates this effect in the case of the Shiga strain and its agglutinative serum.

When the specific effects of all the agglutinative sera upon the cataphoretic charges of their homologous organisms are plotted upon the same chart (Fig. 3), the explanation of this paradoxical behaviour becomes evident. Reference to this figure brings out the fact that all the organisms other than the dysentery strains have, under the conditions of the experiment, high or relatively high charges (23 to 40 millivolts) when untreated with serum; while the three dysentery strains possess relatively low charges (3 to 5 millivolts). It will be seen that in the case of the first group cited there is specific charge reduction, quantitatively related to the agglutinative titer, and that in the second group there is a similar charge elevation. In all cases, however, no matter what the initial charge (range is 5–40 millivolts) high concentrations of serum (over 1:50), bring the charges on all bacteria to a *common potential level* (8–14 millivolts).

In other words, when different bacteria are treated with homologous agglutinative sera in high concentration, they behave cataphoretically alike, irrespective of their previous differences in charge. This suggests that they are alike because they have a similar surface coating. To put it in another way, when bacteria are sensitized by agglutinative sera, a film of agglutinin (globulin) is formed on their surfaces and such sensitized bacteria, in the sense of Loeb's protein-coated particles discussed above, all behave cataphoretically like particles of globulin.

It is worth calling attention, at this point, to the work of Northrop and Freund (26) on the agglutination of red blood cells. They have

found that the potential agglutination zone of unsensitized cells is low (below 6 millivolts) and that, when sensitized the critical point is raised to about 12 millivolts. This is approximately the critical point for globulins, as will be seen below. Accordingly, the difference between the unsensitized and the sensitized cells may well be accounted for on the assumption that the latter are coated with specific antibody (globulin).

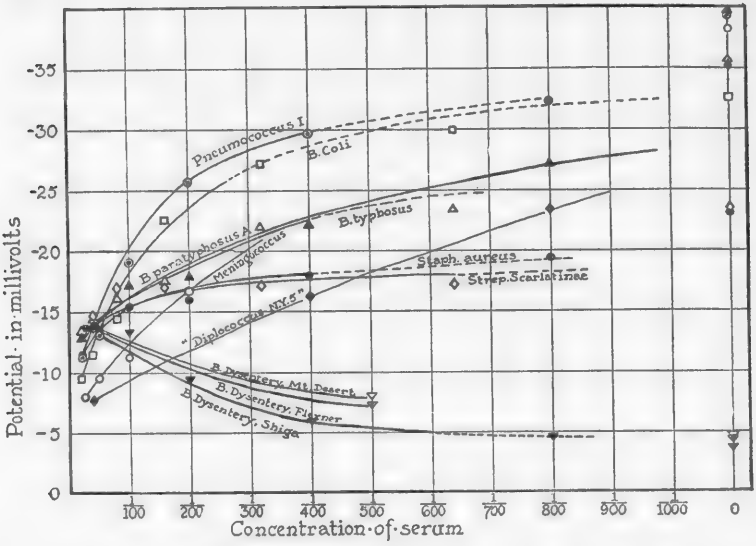

FIG. 3. Effect of eleven specific agglutinating sera upon the potential and agglutination of their homologous organisms. These experiments were done over a period of about 2 years and three different cataphoretic cells were utilized in the work. All experiments in M/200 glycocoll-phosphate-acetate buffer mixture, pH 7.0.

That the conception of sensitization as specific coating with globulin is correct is made still more clear by the evidence afforded in the following experiments.

The cataphoretic behaviour of sensitized Type I pneumococcus was compared with that of the globulin from the homologous serum as follows: The euglobulin of the serum was precipitated by 1:20 dilution with distilled water. The particles thus obtained have protective

antibody (Felton (27)), and agglutinin, as demonstrated by control experiments; they are readily visible microscopically and are thus susceptible of study in an electrophoretic cell. The bacteria were sensitized by treatment with 1:20 immune serum. Unsensitized organisms and organisms treated with 1:20 normal serum were used as controls. The four sets of particles were now added to varying concentrations (M/25–M/800) of glycocoll-phosphate-acetate buffer (21) of pH 7.0, and their charges determined. The results are shown in Fig. 4. The upper curve is that of the untreated organisms, and

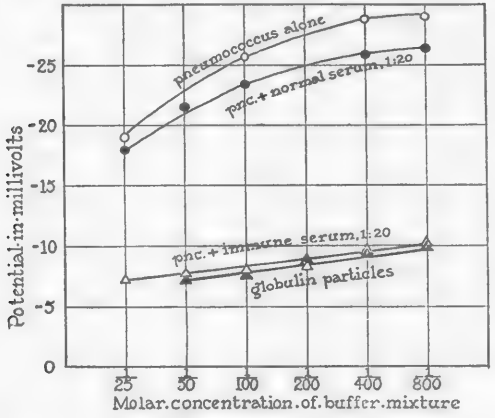

FIG. 4. Effect of glycocoll-phosphate-acetate buffer, pH 7.0, in varying molar concentration, upon potential of Pneumococcus Type I, (*a*) alone, (*b*) treated with 1/20-normal and (*c*) with 1/20 specific serum, and (*d*) of globulin particles obtained from the specific serum.

the next, that of those treated with normal serum, showing the usual slight charge reduction. The two lower curves are those with which we are chiefly concerned. It will be seen that the curve of the sensitized organisms is practically identical with that of the globulin particles. That is, the former are acting cataphoretically as if they were globulin, a fact which can be explained only on the assumption that they are coated with globulin.

The cataphoretic charges of sensitized organisms were next compared with those of particles of denatured pseudoglobulin in solutions

of varying hydrogen ion concentration. The bacteria were colon bacilli treated with 1:20 homologous agglutinating serum, centrifuged, and then resuspended in the solutions in which they were to be tested. The pseudoglobulin, non-specific, was denatured by bringing it, in dilute solution, to the boiling point; the resultant flocculant mass was centrifuged, divided into small homogeneous particles in a mortar, and added to the solutions of varying pH. Unsensitized colon bacilli were tested similarly for comparison. The results appear in Fig. 5.

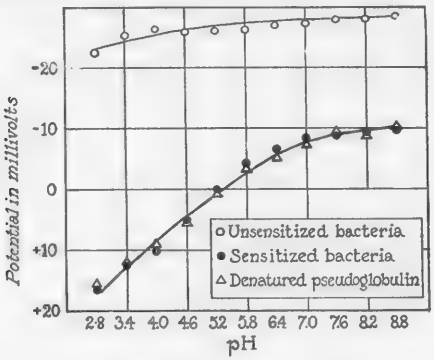

FIG. 5. Effect of varying pH upon potential of unsensitized and sensitized colon bacilli and of particles of denatured pseudoglobulin. Experiment with glycocoll-phosphate-acetate buffer, M/25. Agglutination of sensitized bacteria and pseudoglobulin was complete throughout and was absent in case of unsensitized colon bacilli.

It will be observed that the cataphoretic behaviour of the sensitized bacteria is quite unlike that of the unsensitized organisms, but it is practically identical with that of the denatured pseudoglobulin particles. In other words, the sensitized organisms behave cataphoretically as if they are particles of globulin. A finding again accounted for by the assumption that the organisms are coated with agglutinin.

It will be noted also, from this chart, that the isoelectric point of the sensitized bacteria is the same as that of the pseudoglobulin, which is the accepted one of approximately pH 5.3. This is in accord with the findings of Coulter, Northrop and De Kruif, and Eggerth and Bellows, already cited.

The evidence presented above seems to lead reasonably safely to the conclusion that the first step in specific bacterial agglutination is a selective film formation at the surface of the organism by the globulin of the antibody, and that the changes in charge on the bacteria are the result of this coating. In high concentrations of homologous agglutinating serum, the coating by globulin is complete and the organisms behave exactly like particles of globulin. When the serum is dilute, the degree of coating is less and the charges observed represent the potential difference between the surrounding medium and a combination of bacterial surface and agglutinin film.

What degree of coating is essential for agglutination of the bacteria we are not prepared to say, as the data for such estimation are difficult to obtain. Northrop and De Kruif believed from their experiments, that if film formation could be assumed, it could be calculated that agglutination occurs when the surface is one-eighth covered. From examination of our results with sera having a high agglutinative titer, it is our impression that the amount of coating required may well be less than this.

The further interpretation of the part played by this selective coating of the bacteria in specific agglutination is based largely upon certain observations and conclusions of Loeb (10, 18, 22) in his work with collodion particles and proteins. It is necessary therefore, to consider briefly those portions of his work that bear upon our problem.

Loeb treated collodion particles with various proteins, egg albumin, gelatin, casein, edestin, and found that a durable film of these proteins is formed at the surface of the particles. He showed that such coated particles now behave cataphoretically as if they are no longer collodion particles but are particles of the protein used.

He observed further, that in respect of their stability in suspension, the coated particles (except those treated with gelatin) no longer behave like soluble native proteins which are precipitated only by very strong salt, but act like denatured proteins, which are relatively unstable and are easily thrown out of suspension by electrolytes in low concentration. That is to say, the chemical forces of attraction between the "aqueous" groups of the protein molecule and the water are destroyed in the film formation, as is the case when protein is denatured by boiling, and the stability of the coated particles depends only, as does that of denatured proteins, upon the electrical double layer surrounding each particle (*i.e.*, the charge). To recapitulate, collodion particles coated with protein no longer act like collodion particles, but behave as if they are particles of denatured protein, and flocculate

as soon as their charge is reduced by electrolyte to a critical point of about 13 millivolts.

It is important to note, that in the case of particles of denatured protein, the cohesive force (in the sense used for bacteria, by Northrop and De Kruif) is practically insusceptible to the reduction effect of high concentrations of salt. This is true also for protein-coated particles.

These findings of Loeb supply the reason for the observed fact that sensitized bacteria behave like particles of denatured protein. That is, when the organism is coated with agglutinin and assumes the character of a globulin particle, by virtue of this very film formation, it takes on the character of denatured globulin. Because of this similarity, Tulloch, as noted above, believed that sensitization is akin to denaturation of bacteria. However, the bacterial surface itself is not "denatured," but the film of globulin formed at its surface assumes the characteristics of denatured protein, and the coated organism now acts like a particle of denatured globulin. The same author's analogy between native protein and unsensitized organisms, also noted above, is probably incorrect, as the behaviour of the former is dependent upon true solubility and the failure of the latter to flocculate is due to reduced cohesive force, as shown by Northrop and De Kruif.

The observation of Northrop and De Kruif that the cohesive force of sensitized bacteria is protected against reduction by strong salt is also accounted for. The explanation lies in the fact that the surface film of globulin, having the character of denatured protein, is insusceptible to this depressing effect, and that while electrolytes may reduce the charge on the coated particles, they do not affect their cohesive force, and agglutination is determined solely by reduction of the potential to the critical 15 millivolt level.

Before proceeding to full application of these findings of Loeb to specific agglutination, it seemed necessary to prove that the behaviour of globulins with collodion particles is similar to that of the proteins used by Loeb. Accordingly, certain of his experiments were repeated with pseudoglobulin.

The procedure followed was essentially that described by Loeb (10, 18, 22). Collodion particles were coated with isoelectric pseudoglobulin. Particles of denatured pseudoglobulin were prepared by bringing a dilute solution to the boiling point, centrifuging the floc-

culant mass, and dividing it into suitable homogeneous particles in a mortar. The effect of three electrolytes, NaCl, $K_4Fe(CN)_6$, and $CeCl_3$, upon the charge and agglutination of untreated collodion particles, coated particles, and particles of denatured pseudoglobulin was determined at pH 3.0.[5] The results, shown in Fig. 6, demonstrate the fact that pseudoglobulin behaves in similar fashion to the proteins used by Loeb. Reference to the figure shows that collodion particles treated with pseudoglobulins no longer behave like the untreated

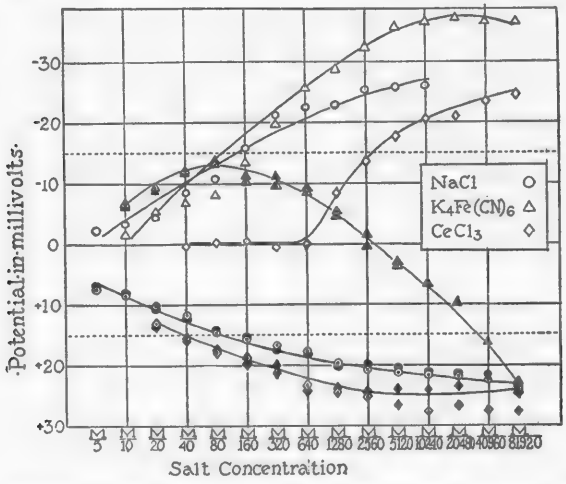

FIG. 6. Influence of NaCl, $K_4Fe(CN)_6$, and $CeCl_3$ upon potential and agglutination of collodion particles (blank symbol), collodion particles coated with pseudoglobulin (symbol with solid center), and particles of denatured pseudoglobulin (solid symbol). Experiment at pH 3.0. Critical potential agglutination zone was between +13 to +15 millivolts and −13 to −15 millivolts. In this zone all particles flocculated out of suspension.

particles, but assume the cataphoretic charges and the critical potential agglutination level (14 millivolts) of the denatured protein. That is to say, they now behave, in these respects, as if they are particles of denatured pseudoglobulin.

[5] The experiments were conducted at this pH because Loeb believed that coating occurred only on the acid side of the isoelectric point of the protein used. Dr. Northrop, in a personal communication, states that evidence has been obtained in his laboratory that coating occurs on the alkaline side as well.

SUMMARY.

From the foregoing evidence, specific bacterial agglutination may be conceived of as follows: When bacteria are mixed with their homologous agglutinative sera, specific union between organism and agglutinin occurs. This interaction consists of specific coating of the bacteria by globulin. By virtue of the fact, noted by Loeb and illustrated in Fig. 6, that protein film formation gives the coated particle the characteristics of denatured protein, the bacteria now take on the character of particles of denatured globulin. Particles of denatured protein flocculate whenever their charge is reduced by electrolyte to a critical level lying somewhere between 12 and 14 millivolts. This is true even when the salt is strong, as, unlike bacterial particles, their cohesive force is not readily depressed by salt. The sensitized bacteria, now being essentially particles of denatured protein, likewise agglutinate as soon as their charge is reduced by electrolyte to this potential level.

CONCLUSIONS.

1. In the process of sensitization by agglutinating serum bacteria are coated selectively by the globulin of the antibody.

2. This film formation causes the bacteria to take on the characteristics of particles of denatured globulin.

3. Subsequent agglutination of the coated bacteria follows the laws governing the flocculation of particles of denatured protein by electrolytes.

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IMMUNOLOGICAL RELATIONSHIPS OF ENCAPSULATED AND CAPSULE-FREE STRAINS OF ENCAPSULATUS PNEUMONIÆ (FRIEDLÄNDER'S BACILLUS).

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In a preceding paper (1) evidence was presented that bacilli of the Friedländer group are separable into sharply defined and specific types. Of 30 strains employed in the study, three specific types and a heterogeneous group were demonstrated by agglutination, agglutinin adsorption, protection, thread and precipitin reactions. These types have been designated Type A, Type B, and Type C, while the remaining unclassified strains were placed in a tentative group, Group X. In the light of the present studies, the difficulties encountered in previous attempts to interpret the immunological reactions of Friedländer's bacillus appear to be due in large measure to the failure to distinguish in the cell the type-specific from the species-specific antigen; and the failure to recognize in immune serum the two distinct antibodies provoked by the respective antigens. The application of this concept to the Friedländer group was suggested by studies carried out in this laboratory on the immunological relationships of the cell constituents of Pneumococcus (2–7). Briefly, this concept involves two separable and distinct antigens—the one a soluble specific substance (now identified as a carbohydrate) which endows the organism with type specificity; the other, a protein substance which exhibits only the common and undifferentiated characters of the species.

Since the relation of the capsular material of Friedländer's bacillus to type specificity has been demonstrated (1, 8, 9), it seemed of importance to determine the immunological relationships of encapsulated and capsule-free strains of this organism. For this purpose, S and R varieties of the bacterial cell were obtained as will be de-

scribed later. It was observed that while S cells were encapsulated, the R forms no longer possessed capsules.

The nomenclature of S and R was first used by Arkwright (10) to describe biological variations in single strains of bacteria. His observations included the occurrence of two forms of colonies in old cultures of bacilli of the intestinal group. The S organisms grew in colonies with a smooth surface while the R strains presented colonies with a rough and irregular surface. The terms S and R have been universally adopted to describe similar changes in other species of bacteria. Observations on encapsulated organisms, however, fall mainly within the Pneumococcus group. With these organisms, it has been shown that accompanying the change from S to R, there is a loss of agglutinability by specific sera, loss of capsule, and attenuation of virulence (10–19).

Cultures of Friedländer's bacillus, also, have been separated by earlier workers (20–24) into S and R components, although such a designation was not in use at the time. The method of decapsulation as devised by Porges (25) must also be considered of the same order. More recently, Friel (13) and Hadley (26) have reported the occurrence of the two varieties in individual cultures of Friedländer's bacillus and have attributed to them the accepted characteristics of S and R forms.

In this study, the criteria for the conversion of S organisms to R forms have been: (1) loss of capsule and mucoid characteristics, (2) loss of agglutinability in type-specific sera, (3) attenuation of virulence, and (4) the development of colonies which present under the microscope a rough and irregular surface. In the case of R cells derived from Type C, loss of virulence was not an accompanying change since none of the Type C strains were virulent for mice.

The present report deals with the immunological reactions of encapsulated and capsule-free strains of Friedländer's bacilli and anti-S and anti-R sera, and the serological behavior of encapsulated bacilli after removal of capsules by the chemical method of Porges.

Methods.

Loss of Capsule Formation Induced by Growth in Vitro.—The non-encapsulated or "R" strains employed in this study were derived from pure S strains. An S strain representative of each of the three fixed types and one strain chosen from Group X were transplanted daily in broth to which had been added 10 per cent of homologous immune serum. Plates, streaked at the time of each transplant, were examined microscopically after 18–24 hours incubation, as recommended by Reimann (18). Within 6–10 transplants, plate cultures were obtained in which a

number of R colonies were observed. Single, typical R colonies were then transplanted into plain broth and the resultant R culture showed loss of capsule and attenuation of virulence. In each instance, the R culture failed to kill white mice, in doses of 0.5 cc., while the parent S strain from which the R had been derived regularly killed within 48 hours at a dilution of one ten-millionth cc.

Destruction of Capsule by Chemical Means.—The method devised by Porges was used. S strains were grown on agar slants and the organisms were washed off and suspended in salt solution. The suspensions were made acid with one-fourth volume N/4 HCl and heated at 80°C. for 15 minutes. The suspensions were then cooled immediately under tap water and neutralized with an equivalent quantity of N/4 NaOH. The exposure to heat was varied from 60°C. to 100°C. and from 10 minutes to 30 minutes without appreciable differences. No spontaneous clumping of the treated organisms was experienced when freshly prepared suspensions were used. After standing in the ice chest several days, however, preparations from two different strains became granular.

The method of immunization and the reaction of agglutination, precipitin and protection tests were conducted in the manner described in the preceding paper (1).

EXPERIMENTAL.

I. Immunological Reactions of "S" (Encapsulated) Strains in Immune Sera.

(*a*) *Anti-S Sera.*—It has already been shown in the preceding paper (1) that antisera prepared by immunization with encapsulated strains possess type-specific immune bodies, which agglutinate the encapsulated organisms and precipitate the soluble specific substance derived from them. In addition, such sera confer specific protection upon white mice against infection with strains of homologous types.

(*b*) *Anti-R Sera.*—Since the anti-R sera were prepared against strains of organisms which were capsule-free and which had lost the function of elaborating the type-specific soluble substance, it was to be anticipated that these sera would be lacking in type-specific antibodies. Definite proof of this, however, was obtained by determining the agglutination of S strains in anti-R sera. For purposes of comparison, the specific reactions of these same strains in anti-S sera of the homologous type were included. The results presented in Table I show conclusively that encapsulated strains are not agglutinated by sera prepared by immunization with the capsule-free (R) variants. This is true even though the organism employed for

immunization was derived from the S strain later used for agglutination.

TABLE I.

Agglutination of Friedländer S Strains by Anti-R Sera.

Antigen encapsulated strain	Anti-R sera									Anti-S sera			
	Type A			Type B			Group X			A	II	C	X
	*1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10	1:5	1:5	1:5	1:5
Type A	−	−	−	−	−	−	−	−	−	++++	−	−	−
" B	−	−	−	−	−	−	−	−	−	−	++++	−	−
" C	−	−	−	−	−	−	−	−	−	−	−	++++	−
Group X	−	−	−	−	−	−	−	−	−	−	−	−	++++

* The figures in this row represent ultimate dilution of serum.

++++ indicates compact disk agglutination with clear supernatant; −, no reaction.

TABLE II.

Precipitation of the Soluble Specific Substance of Friedländer's Bacillus by Anti-R Sera.

Antiserum	Soluble specific substance of Friedländer's bacillus											
	Type A						Type B					
	*2	20	50	100	250	500	2	20	50	100	200	500
Type A (R)	−	−	−	−	−	−	−	−	−	−	−	−
" B "	−	−	−	−	−	−	−	−	−	−	−	−
Group X "	−	−	−	−	−	−	−	−	−	−	−	−
Type A (S)	−	+	++	++	+	−	−	−	−	−	−	−
" B "	−	−	−	−	−	−	−	++	+++	++++	+++	++
Normal	−	−	−	−	−	−	−	−	−	−	−	−

* The dilutions are expressed in thousands.

++++ indicates heavy, compact, disk precipitate; +++, marked disk precipitate; ++, thin, film-like scale; +, ground glass turbidity.

Further evidence of the lack of type-specific antibodies in anti-R sera was sought by the precipitin reaction. Solutions of the isolated soluble specific substances of Types A and B were tested against three anti-R sera. As demonstrated in Table II, these sera contained no pre-

cipitins for the type-specific substances just as in the preceding experiment they were shown to possess no agglutinins for the encapsulated cells from which these substances were derived.

It has been shown in the previous communication that anti-S sera contain antibodies which afford specific protection in mice against

TABLE III.

Protection Offered by Anti-R Sera against Infection by Type A, Friedländer's Bacillus.

Type A encapsulated culture	Anti-R sera derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
	cc.		*cc.*		
.001	.2	D. 16 hrs.	.2	D. 20 hrs.	
.0001	.2	" 19 "	.2	" 20 "	
.00001	.2	" 39 "	.2	" 22 "	D. 24 hrs.
.000001	.2	S.	.2	" 65 "	" 39 "
.0000001					" 65 "

S. indicates survival; D., death, the numerals representing the number of hours before death occurred.

TABLE IV.

Protection Offered by Anti-R Sera against Infection by Type B, Friedländer's Bacillus.

Type B encapsulated culture	Anti-R sera derived from				Virulence controls
	Type A		Type B		
	Amount	Result	Amount	Result	
	cc.		*cc.*		
.001	.2	D. 15 hrs.	.2	D. 15 hrs.	
.0001	.2	" 15 "	.2	" 15 "	
.00001	.2	" 39 "	.2	" 24 "	D. 16 hrs.
.000001	.2	" 22 "	.2	" 65 "	" 39 "
.0000001					" 39 "

infection with virulent encapsulated bacilli of homologous types. Accordingly, an analysis of anti-R sera was made to determine the presence of protective properties. The results of these determinations are given in Tables III and IV. An anti-R serum prepared by immunization with a degraded Type A organism affords no measura-

ble protection against infection with an encapsulated (S) strain of Type A or Type B. Similarly, an anti-R serum prepared by immunization with a capsule-free organism derived from Type B offers no protection against infection with virulent strains of either of these specific types.

In summary, then, the foregoing experiments show that antisera prepared by the immunization of rabbits with degraded, capsule-free strains of Friedländer's bacillus (R forms) are devoid of specific agglutinins, precipitins, and protective antibodies for the virulent, type-specific, encapsulated bacilli. Anti-R sera, therefore, exhibit none of the type-specific reactions which characterize anti-S sera.

II. Immunological Reactions of "R" (Non-Encapsulated) Strains in Immune Sera.

(*a*) *Anti-S Sera.*—The anti-S sera used for the determination of specific types (1) were prepared in such manner as to avoid or at least minimize the concurrent presence of the common specific antibody. As was pointed out in the preceding paper, this may be accomplished most successfully by using young cultures of encapsulated organisms and by avoiding prolonged immunization. It was considered of interest to determine the agglutinative action of the type-specific sera against R strains of different origin. The results of these tests are given in Table V. It is seen that an immune serum prepared against an encapsulated strain of Type A contains only a small amount of the species antibody. In addition, this antibody is shown to be equally operative against four R strains, each of which in turn had been derived from an S organism of a serologically different type. The three anti-S sera of Type B, Type C, and Group X contained only minute and, for practical purposes, negligible traces of the R or common species antibody. Thus, further confirmation is advanced for the concept that the R organisms, devoid of capsules, are no longer type-specific.

(*b*) *Anti-R Sera.*—Evidence has been presented that capsule-free strains of Friedländer's bacillus are lacking in the ability to engender type-specific antibodies. However, they stimulate the formation of antibodies which react not only with the particular strain used for immunization, but with all other R forms regardless of the type from

which they were derived. This fact is revealed in the cross-reactions of agglutination presented in Table VI in which all R strains are shown to be reciprocally agglutinated by each of the different anti-R sera.

The agglutination of the R strains in anti-R serum is characteristically different from the agglutination of S strains in anti-S serum of the homologous type. It will be recalled that with S strains, the reaction results in the formation of a compact, firm disk of agglutinated bacteria and occurs almost immediately in the more concentrated dilutions of the serum. The R organism, on the other hand, agglutinates very slowly, and in the higher concentrations of serum forms a fluffy precipitate; while in greater dilutions of the serum a fine, granular agglutination occurs which is difficult to read without a

TABLE V.

Agglutination of Friedländer R Strains by Anti-S Sera.

Antigen capsule-free strain	Anti-S sera											
	Type A			Type B			Type C			Group X		
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10
Type A	++	+	+	±	−	−	−	−	−	−	−	−
" B	++	+	+	±	−	−	−	−	−	−	−	−
" C	+	+	+	±	−	−	+	−	−	+	−	−
Group X	++	+	+	±	−	−	+	−	−	+	−	−

lens. The agglutinins in anti-R sera, which react with capsule-free strains, bear a marked similarity to the "fine flaking" agglutinins for the H-form of *proteus* and typhoid bacilli described by Felix (27) and to the somatic agglutinins for the non-motile forms of hog-cholera bacillus reported by Orcutt (28). Furthermore, the agglutinin titre of anti-S sera is low (1:40–1:80) while the agglutinin titre of anti-R sera is high (1:2500).

Corroborative evidence of the serological identity of R strains was gained by the agglutinin adsorption test. Each anti-R serum was adsorbed with heat-killed suspensions of each R strain until all the agglutinins were removed for the adsorbing organisms. The adsorbed sera were then tested for the presence of agglutinins for other R strains. Repeated tests yielded constant results. To avoid un-

TABLE VI.

Cross-Agglutinations of R Strains of Friedländer's Bacillus by Anti-R Sera.

Anti-R sera	Antigen derived from	Final dilution of serum										Normal serum 1:5
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Type A	Type A	++++	++++	++++	++++	++++	++++	++++	++++	+++	+	−
	" B	++++	++++	++++	+++	++	+	+	±	−	−	−
	" C	++++	++++	++++	++++	+++	+++	++	+	−	−	−
	Group X	++++	++++	++++	+++	++	++	+	+	−	−	−
" B	Type A	++++	++++	++++	++++	++++	+++	++	+	+	−	−
	" B	++++	++++	++++	++++	++++	+++	+++	++	++	+	−
	" C	++++	++++	++++	++++	+++	+++	++	++	+	−	−
	Group X	++++	++++	++++	++++	++++	+++	+++	++	++	+	−
Group X	Type A	++++	++++	++++	++++	++++	+++	++	++	+	−	−
	" B	++++	++++	++++	++++	+++	++	++	+	−	−	−
	" C	++++	++++	++++	++++	+++	+++	++	++	+	−	−
	Group X	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	−

++++ indicates complete agglutination with flocculent sediment and clear supernatant; +++, almost complete, supernatant clouded; ++, marked agglutination; +, slight agglutination; −, no agglutination.

necessary repetition, a single typical protocol is given in Table VII. It is evident that the adsorption of an anti-R serum with any R strain removes from the serum agglutinins for the homologous organism as well as for heterologous strains. It is apparent, therefore, that immunologically all R cells are identical, as tested by the reactions of agglutination and agglutinin adsorption.

TABLE VII.

Agglutinin Adsorption.

Results of Agglutination with Anti-R Serum (Type B) after Adsorption by R Strains Derived from Homologous and Heterologous Types of Encapsulated Organisms.

Antigen capsule-free strain from	Anti-R serum (Type B) after adsorption with R strains derived from															
	Type A				Type B				Type C				Group X			
	*20	50	100	500	20	50	100	500	20	50	100	500	20	50	100	500
Type A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Group X	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* Figures represent ultimate dilution of serum.

III. Immunological Reactions of "S" (Encapsulated) Strains after Decapsulation by Porges' Method.

Appreciating the difficulties in the agglutination of Friedländer's bacillus, Porges devised a method of removing the capsule by weak acid in order to render strains more antigenic and better agglutinable. To test the effect of decapsulation on the serological behavior, suspensions of encapsulated bacilli representative of each of the specific types were subjected to the Porges technique. The cells were then tested for agglutinability in sera prepared by immunization with encapsulated and capsule-free organisms, respectively. For purposes of comparison, a strain of *Encapsulatus granulomatis* was included in the experiment.

(*a*) *Anti-S Sera.*—The results obtained with anti-S sera are summarized in Table VIII. It is evident that encapsulated organisms

which are heated in the presence of acid are not agglutinated appreciably in purely anti-S sera of types which are serologically different from that yielding the decapsulated cells. Agglutination in such sera depends upon the presence of species antibody. In antisera of the parent strains, however, there is a definite precipitin reaction which is referable to the presence of unhydrolyzed soluble specific substance. In this connection it will be recalled (1, 9) that the capsular material of Friedländer's bacillus is precipitated by anti-S serum of the homologous type. Since the method of Porges strips the bacillus of the capsule with apparently only partial hydrolysis, it is not surprising that precipitation of the soluble specific substance is observed.

TABLE VIII.

Agglutination by Anti-S Sera of Suspensions of "S" Strains Decapsulated by Porges' Method.

Antigen derived from	Anti-S serum								
	Type B			Type C			Group X		
	1:1	1:5	1:10	1:1	1:5	1:10	1:1	1:5	1:10
Type A.....	+	−	−	+	+	−	+	+	−
" B.....	*+++	++	+	+	+	−	+	+	−
" C.....	+	+	−	*+++	++	+	++	+	−
Group X.....	+	+	−	+	+	−	*+++	++	+
Gran.....	+	+	−	+	±	−	++	+	−

* Typical "S" reaction.

(*b*) *Anti-R Sera.*—In anti-R sera, however, chemically decapsulated suspensions show a marked agglutination to a high dilution of serum and the reaction gives the characteristic appearance of R agglutination. The suspensions react equally well in all anti-R sera regardless of their type derivation. (Table IX.)

It is interesting to note that a closely related organism, *Encapsulatus granulomatis*, when subjected to the same conditions, is also agglutinated by anti-R sera. This explains in a measure the confusion experienced with allied encapsulated Gram-negative organisms. The species antigen of the Friedländer's bacillus is related to some extent to the species antigen of what have been considered allied organisms.

To recapitulate, encapsulated strains (Friedländer's bacillus) are transformed into "R" strains by heating in acid solution. The transformed cells react identically as "R" cells obtained by cultural methods. They agglutinate in anti-S sera depending upon the presence of species antibody, and in anti-R sera they behave as typical "R" strains.

TABLE IX.

Agglutination by Anti-R Sera of Suspensions of "S" Strains Decapsulated by Porges' Method.

Anti-R sera	Antigen derived from	Dilution of serum				
		1:50	1:100	1:250	1:500	1:1000
Type A	Type A	+++	+++	++++	++++	+++
	" B	++++	++++	++++	+++	++
	" C	+	++	+++	++	+
	Group X	+++	++++	++++	++++	+++
	*Gran.	++	++	+	−	−
" B	Type A	+++	+++	++++	++++	+++
	" B	++++	+++	++	+	−
	" C	++	++++	++	+	−
	Group X	++++	++++	++++	+++	++
	Gran.	+++	++	++	+	−
Group X	Type A	++++	++++	++++	++++	+++
	" B	++++	++++	++	++	+
	" C	++	+++	++++	+++	++
	Group X	++++	++++	++++	+++	++
	Gran.	+++	++	+	−	−

* This organism was isolated from an infection of granuloma inguinale.

DISCUSSION.

"S" strains of Friedländer's bacillus are characterized biologically by mucoid growth in liquid media, by capsule formation, and by exalted virulence. As antigens they give rise in the serum to type-specific antibodies. Consequently, such an immune serum will agglutinate all organisms of the homologous type, afford passive protection in white mice against infection by strains of the same type, and precipitate the soluble specific substance derived from the type strains. The extent of species antibody in anti-S sera is negligible

provided immunization has not been intensive. The experience of former investigators, including our own, has been that in general prolonged immunization, even with capsule-bearing organisms, has yielded a serum so abundant in the species antibody as to obscure type-specific reactions. It is now recognized (7) in this connection that dissociation of the bacterial antigen occurring spontaneously *in vitro* and *in vivo* is a factor to be considered in immunization. That the dissociation observed in this study is due to a cleavage of the specific antigen complex in the animal body rather than a condition of cultural development is substantiated by certain evidence to be presented in a later paper.

"R" strains of Friedländer's bacillus on the other hand are characterized by loss of capsule formation, loss of the elaboration of soluble specific substance, and by attenuation of virulence. The sera of animals immunized to R strains contain only the species antibody. In other words anti-R sera agglutinate only capsule-free strains, irrespective of their type antecedent. Furthermore, anti-R sera fail to react with encapsulated organisms. They afford no passive protection against infections with virulent type-specific strains of Friedländer's bacilli, and they do not precipitate the soluble specific substance derived from organisms of homologous or heterologous type.

It is obvious, therefore, that the nature of antibody response in the animal is dependent upon the character of the bacterial cell which is utilized for immunization. Immunization with encapsulated strains engenders type-specific antibodies. On the other hand, a bacterial culture composed of both encapsulated and capsule-free cells, as is often the case, induces both the type-specific and species-specific antibodies. The predominance of the S or R component determines the predominance of the one or the other antibody. In either case confusing cross-agglutination reactions will be encountered, as they have been in the past, which are difficult of interpretation unless cognizance is made of the underlying principles. Immunization which is effected with cells devoid of capsules, as advocated by former workers (20, 21, 25), gives rise to only the species antibody which exhibits none of the type relationships. Similarly, in the agglutination reaction will be reflected the composition of a strain.

The studies on the cell constituents of Pneumococcus to which reference has already been made reveal a striking similarity in the immunological behavior of the two encapsulated races. There has been observed in both species the occurrence of encapsulated, virulent organisms which differ serologically and which undergo degradation to such an extent as to become capsule-free, avirulent, and serologically undifferentiated.

These principles find a remarkable analogy even among flagellated bacteria as is indicated by Smith and Reagh (29), Orcutt, and Felix. These authors have shown independently that certain differences are demonstrable in the serological behavior between flagellated and non-flagellated organisms of the same strain. These differences have been related to the presence of two antigens, the flagellar or ectoplasmic, and the somatic or endoplasmic antigen.

CONCLUSIONS.

1. "S" strains of Friedländer's bacillus produce capsules, soluble specific substance, and are of exalted virulence. "S" strains are type-specific and react with only the type-specific antibodies of the homologous types.

2. Immunization with "S" cells induces the formation of antibodies which agglutinate type specifically, precipitate the corresponding soluble specific substance, and protect white mice against infection caused by organisms of the same type.

3. "R" strains of Friedländer's bacillus produce no capsules, produce no soluble specific substance, and are not pathogenic. "R" strains are serologically undifferentiated from each other and react with only the species antibodies.

4. Immunization with "R" cells induces antibodies which do not agglutinate encapsulated organisms, do not precipitate soluble specific substance, and do not afford protection against infection by Friedländer's bacillus. Anti-R serum contains only the species antibody which reacts with any capsule-free organism regardless of its type origin.

5. Decapsulation of "S" cells by heat and acid chemically converts a type-reacting organism into a species-reacting organism.

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ETIOLOGY OF OROYA FEVER.

III. The Behavior of Bartonella bacilliformis in Macacus rhesus.

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Plates 22 to 24.

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As reported in a previous paper,[1] a strain of *Bartonella bacilliformis* isolated from the blood of a fatal case of Oroya fever when inoculated into young *Macacus rhesus* monkeys induced a protracted course of intermittent fever, resembling that observed in some human cases of Oroya fever. The microorganism was demonstrated in the red cells of these animals and recovered in culture, but the clinical manifestations in monkeys differed from those of the human disease in that none of the animals showed the severe anemia so characteristic of Oroya fever. It was also shown that intradermal inoculation of the cultures into the shaved skin of the eyebrows of the monkeys gave rise to a granulomatous nodule rich in capillaries, in which the microorganisms could be demonstrated by section and by culture. The nodules presented a marked resemblance to those of verruga peruviana.

Further study of the behavior of *Bartonella bacilliformis* in *rhesus* monkeys, particularly with reference to the effects of passage strains, has brought to light a number of additional facts, which throw some light on the variety of manifestations of *Bartonella* infection in man.

The protocols are presented in chronological order, and the extraordinary variability in response to inoculation of the parasite is illustrated by all of them. The severity of infection in most instances is referable probably to enhanced virulence of the parasite through adaptation to the animal. In Monkey 5 we have an example of severe systemic infection induced by local inoculation. In Monkey 6 intra-

[1] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

venous and intraperitoneal inoculation gave rise to a fatal systemic infection, which was not accompanied by local lesions, and which, except for the slight degree of anemia present, resembled a case of simple Oroya fever; the fever was severe (104–105°C.) and continuous, and the parasites were present in the blood in high titer and also in the lymph glands, spleen, and bone marrow. Monkey 7 was inoculated intradermally and intravenously with both passage virus and cultures, and the reaction was constitutionally as well as locally severe; *Bartonella bacilliformis* was isolated from blood diluted 1:10,000,000, from nodular tissue diluted 1:10,000, and also from the lymph glands, spleen, and bone marrow. Monkey 8, on the other hand, while receiving considerable amounts of passage virus both locally and intravenously, manifested no local and only mild systemic symptoms. Monkey 18 is of special interest as an illustration of a severe type of verruga such as may result from the inoculation of monkeys with human verruga tissues,[2, 3] while the striking fact brought out in the case of Monkey 25 is the simultaneous occurrence of severe symptoms of both verruga and Oroya fever, typical verruga nodules appearing spontaneously at sites remote from those of local inoculation. These appearances had not been observed by previous investigators in monkeys inoculated with human verruga tissues.

Quantitative estimates of the number of *Bartonella bacilliformis* present at any given time in the blood are expressed in terms of the highest dilution of the blood from which cultures of the organism could be obtained. The culture method is the only satisfactory test of the presence of the parasite. The use of monkeys is obviously not practicable for quantitative determination and would not, moreover, be reliable because of the variation in susceptibility to *Bartonella* infection. Careful microscopical examination of stained blood films was not, of course, neglected, but when one considers that citrated blood of as high titer as 1:100,000 would show only 1 cell invaded by the parasite among 3,000 cells (1 cc. of citrated blood containing approximately 3 billion red cells), the difficulties of this method of demonstrating the organism become evident.

[2] Jadassohn, G., and Seiffert, G., *Z. Hyg. u. Infektionskrankh.*, 1910, lxvi, 247.

[3] Mayer, M., Rocha Lima, H., and Werner, H., *Münch. med. Woch.*, 1913, lx, 739.

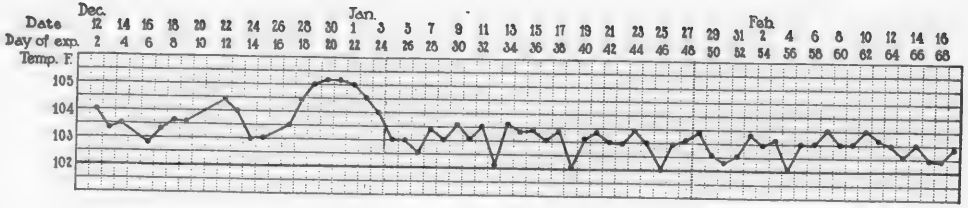
The Invasion of the Blood by B. bacilliformis after Intradermal Inoculation.

The ease with which *Bartonella bacilliformis* may enter the general circulation after intradermal inoculation is illustrated in the case of *M. rhesus* 5.

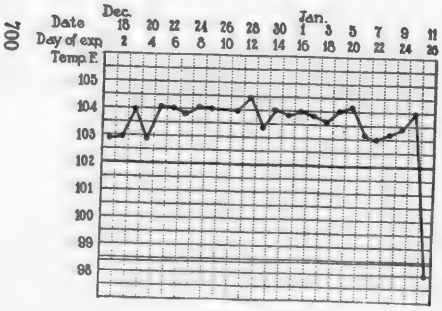
M. rhesus 5 (Text-fig. 1). A young monkey, about 2,000 gm.—a size which has been found most satisfactory for experimental work with *Bartonella bacilliformis*—was inoculated, Dec. 11, 1925, intradermally on the shaved left eyebrow, with 0.2 cc. of a saline suspension of pooled cultures which on dark-field examination showed 3 to 4 motile microorganisms per field. The mixture included 1 cc. of condensation water from a 9 day old culture on a blood agar slant, derived from the original human blood, and 0.3 cc., in 20 cc. of 0.9 per cent saline, of a 10 day old culture, derived from the blood of *M. rhesus* 1, and grown on leptospira medium at 25°C. Cultures made with the blood withdrawn on that date (11 days after inoculation) revealed the presence of *Bartonella bacilliformis* in a titer of 1:100,000, and in stained preparations the parasites were demonstrated in small numbers in the erythrocytes. Cultures made on Dec. 28, 1925, and on Jan. 5, 12, and 25, 1926, also yielded positive results. The animal showed the highest fever during this period, the temperature being continuously at 105°F. from Dec. 29 to Jan. 1. The last two specimens of blood had a titer of only 1:10, and the parasites could no longer be demonstrated in stained smears, hence the number of organisms in the blood stream had diminished rapidly within a period of 24 days. The site of intradermal inoculation on the left eyebrow, however, began to show induration nearly 42 days after injection; on Jan. 22 it was about 2 × 3 mm. in area and was slightly raised; on Feb. 1 it measured 6 × 6 mm., and on Feb. 4 about 9 × 9 mm. It was at first pale and semitransparent, but firm to the touch; then it became a deep rose-red and finally cherry-like in form and color. During the following 10 to 12 days it remained nearly stationary, but later it showed slight excoriation at the apex and was removed* for transfer, culture, and histological studies. It proved to be infective for *rhesus* monkeys, yielded a pure culture of *Bartonella bacilliformis*, and on section showed the characteristic endothelial proliferation with intracellular localization of the organism.

Blood cultures made on Feb. 9 and 17 yielded growth of *Bartonella bacilliformis* in 0.1 cc. of a 1:10 dilution. At no time after subsidence of the early violent febrile reaction had the animal's temperature been higher than 103.6°F. The animal was killed under ether anesthesia on Feb. 17, 1926, 68 days after inoculation, in order that the distribution of the microorganisms and the character of the specific lesions might be ascertained.

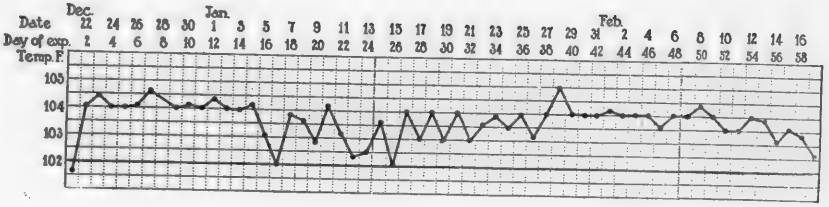
* All operations were performed under ether anesthesia.



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

Autopsy.—Spleen enlarged and granular on surface, culture +. Lymph glands everywhere enlarged, culture +. Heart blood culture +. Bone marrow (femur) reddish, culture +. Lungs normal, culture negative. Liver normal, culture negative. Kidney normal, culture lost because of contamination.

With the cooperation of Dr. J. H. Bauer, erythrocyte counts and hemoglobin estimates[4] were made on several occasions. The results follow:

Jan. 12, 1926 (32 days after inoculation). Erythrocytes 6,169,000. Hemoglobin 82 per cent.

Jan. 25, 1926 (45 days after inoculation). Erythrocytes 5,901,000. Hemoglobin 75 per cent.

Feb. 15, 1926 (66 days after inoculation). Erythrocytes 5,248,000. Hemoglobin 70 per cent.

Feb. 17, 1926 (68 days after inoculation). Erythrocytes 4,856,000. Hemoglobin 70 per cent.

The chronic nature of the infection is clearly indicated by the persistence of the parasites in the peripheral blood, bone marrow, lymph nodes, and spleen. The number of microorganisms demonstrated by stained preparations in the blood corpuscles was never large at any time, even during the febrile or "septicemic" period. There was little sign of anemia until the latter part of the experiment, when the number of red cells seemed definitely to have diminished, notwithstanding the marked fall in temperature and the scarcity of *Bartonella bacilliformis* in the blood at this time.

Variability in the Effect of Inoculation of Bartonella bacilliformis into Macacus rhesus.

The outcome of inoculation of a given pathogenic microorganism must depend in part on the virulence of the strain and in part on the degree of susceptibility of the host. In the case of a newly isolated microorganism the effect of successive animal passages on virulence is problematic; the pathogenic property may be either weakened or enhanced by continued passage. Experiments have just been described in which a second passage of *B. bacilliformis* through *Macacus rhesus* indicated a decided increase in virulence of the organism for this species of animal. The present experiment has to do with the effects of further direct passages in *rhesus* monkeys.

[4] Dr. J. H. Bauer was kind enough to make the blood counts on all the animals of this report. The hemoglobin estimates were made by means of the Sahli hemoglobinometer.

As the protocol shows, intravenous and intraperitoneal inoculation of a young monkey (*M. rhesus* 6) with the citrated blood from infected monkeys of the same species led to an immediate and severe febrile reaction to which the animal succumbed after 3 weeks of continuous fever of about 104°F.

M. rhesus 6 (Text-fig. 2) received 2 cc. of citrated blood from *M. rhesus* 2 intravenously on Dec. 17, 1925, and 5 cc. of a mixture of citrated blood from Monkeys 2, 3, and 4 intraperitoneally on Dec. 18. The temperature rose to 104°F. within 48 hours, dropped to 103° for 1 day, returned to 104°, and remained at about that point until the 20th day. It was 103° on the 21st to 24th days and 104° on the 25th, but on the 26th day it fell suddenly to 98° (subnormal). The animal was cyanotic, cold, and rigid and died during ether anesthesia.

Blood cultures made on the 4th, 12th, 18th, and 26th days after inoculation yielded the following results:

Dec. 21, 1925. Positive with blood diluted 1:100,000.
" 28, " " (untitrated).
Jan. 3, 1926. Positive with blood diluted 1:100,000.
" 11, " " " " " 1:10,000.
" " " " " plasma " 1:10.

The erythrocyte count on Jan. 3, 1926 (17th day), was 4,760,000, hemoglobin 75 per cent. Examination of organs at autopsy revealed enlargement of the spleen and of the lymph glands generally. The other organs appeared normal. *Bartonella bacilliformis* was obtained in pure culture from blood, spleen, and lymph nodes.

The striking phenomena in the foregoing experiment were the absence of localized lesions and the sudden collapse of the animal. The latter can hardly have been due to the insignificant anemia that had developed. The persistent presence of *Bartonella bacilliformis* in large numbers in the blood and organs may have resulted in a toxemia.

That different individual animals react differently to practically the same infective material is demonstrated in the following experiment.

M. rhesus 7 (Text-fig. 3) was inoculated intravenously on Dec. 21, 1925, with 5 cc. of mixed citrate blood from Monkeys 2, 3, and 4. At the same time intradermal inoculations were made on the right eyebrow with 0.2 cc. of an emulsion of the nodule from Monkey 4 and on the left with 0.2 cc. of a young culture, grown on leptospira medium, of blood from Monkey 2. Adjacent areas on each eyebrow were scarified and smeared with the same material which had been injected intradermally into that eyebrow.

In the case of this animal observation was especially directed to (1) the quantitative fluctuation of *Bartonella bacilliformis* in the blood, (2) the course of the local nodular reactions, and (3) the effect of the infection upon the red corpuscles and hemoglobin, this portion of the study being carried out by Dr. Bauer.

The results of blood culture were as follows:

Dec. 24, 1925 (3 days after inoculation). Positive with blood diluted 1:10,000,000.

Dec. 28, 1925 (7 days after inoculation). Positive (not titrated).

Jan. 4, 1926 (14 days after inoculation). Positive with blood diluted 1:1,000,000.

Jan. 18, 1926 (28 days after inoculation). Positive with blood diluted 1:10.
" 29, " (39 " " "). " " " " 1:10.
Feb. 9, " (50 " " "). " (not titrated).
" 17, " (58 " " "). " (" ").

The relatively large number of microorganisms found in the blood withdrawn about 72 hours after intravenous inoculation indicates a strikingly rapid multiplication of the parasites. During the 11 days there would appear to have been a gradual diminution in the number of organisms, and the findings point to an astonishing elimination in the following 2 weeks, since on Jan. 18 *Bartonella bacilliformis* could not be cultivated from blood in dilutions higher than 1:10. The titer was still 1:10, 40 days after inoculation, and the organisms were present in the circulation after 58 days.

In this instance, as in many others, *Bartonella bacilliformis* was demonstrable in small numbers of erythrocytes when the blood titer was high. But as the titer fell to 1:10, examination of film preparations yielded negative results.

The intradermal inoculation of the culture gave rise to a definite nodule (2 × 3 mm.) within 11 days. The lesion gradually increased in size (to 4 × 6 mm.) and had assumed a rose color when it was excised for study on Jan. 8, 1925 (19th day after inoculation). No lesion developed at the site where the culture was inoculated by scarification. The nodule at the site of intradermal inoculation of the nodule emulsion from Monkey 4 became noticeable only after 20 days, and at the same time a small area of induration appeared on the adjacent scarified area. The nodule and indurated area became gradually redder and larger and were removed for study 29 days after inoculation.

Histologically the nodules were the products of infection solely with *Bartonella bacilliformis* and they showed the characteristic localization of the organism in the endothelial cells of the lesions. An interesting difference in the number of organisms present in the nodule and the scarified area (both induced by the emulsion of nodule from Monkey 4) was revealed by titration. The emulsion of the skin lesion yielded growth in a 1:10,000 dilution, while that of the nodule contained sufficient numbers of organisms in a 1:10 dilution to yield a culture, but not in a 1:100 dilution. It may be that the age of the lesion is a factor in this quantitative difference, since the more slowly progressing lesion contained the larger number of organisms.

Blood counts by Dr. Bauer gave the following results:

Jan. 4, 1926 (14 days after inoculation). Erythrocytes 4,400,000.
" 12, " (22 " " "). " 4,620,000. Hemoglobin 75 per cent.
Feb. 17, 1926 (58 days after inoculation). Erythrocytes 5,472,000. Hemoglobin 80 per cent.

It is difficult to draw any conclusion from the fragmentary blood counts. The animal appeared, however, to be in good condition when it was sacrificed on Feb. 17 for examination of various organs (58 days after inoculation).

Autopsy.—The lungs showed a few scattered grayish nodules, the liver a few small grayish spots. The spleen was enlarged and granular; the lymph nodes moderately swollen. The kidneys were normal. *Bartonella bacilliformis* was obtained in culture from the spleen, bone marrow, lymph glands, and heart blood.

In the foregoing experiment we were apparently dealing with a protracted and severe but non-fatal, *Bartonella* infection, with possible visceral involvement. It is of special interest that the local and systemic processes proceeded simultaneously.

That an unusually resistant animal may occasionally be encountered among *Macacus rhesus* monkeys is shown by the following protocol.

M. rhesus 8 was inoculated on Dec. 30, 1925, with an emulsion of the well developed nodule from *M. rhesus* 4.[1] The material was introduced intradermally, by scarification on both eyebrows, and also intravenously (2 cc.). During 50 days of observation there were, roughly speaking, three periods of high fever (Text-

fig. 4). During the first 12 days the temperature rose as high as 106°F., and during the second and third periods it fluctuated about 104° for about 10 days. Although the temperature was 104° on the day following inoculation, the febrile reaction seems to have become definite on the 6th day.

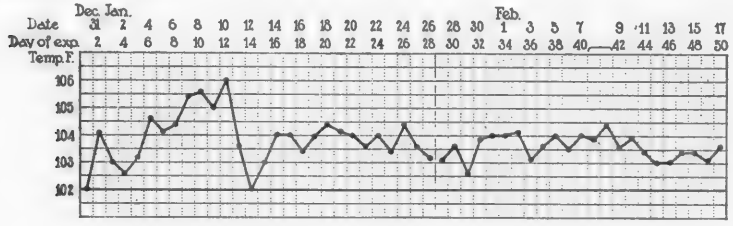
Four blood cultures were made during life and one post mortem, with the following results:

Jan. 4, 1926 (5 days after inoculation). Positive with blood diluted 1:10.
" 8, " (9 " " "). " " undiluted blood.
" 18, " (19 " " "). " " blood diluted 1:1,000.
Feb. 3, " (35 " " "). " " " " 1:10.
" 17, " (49 " " "). Negative.

Blood counts showed no anemia:

Jan. 8, 1926 (9 days after inoculation). Erythrocytes 5,506,000. Hemoglobin 80 per cent.

Feb. 3, 1926 (35 days after inoculation). Erythrocytes 5,414,000. Hemoglobin 80 per cent.



TEXT-FIG. 4.

Feb. 17, 1926 (49 days after inoculation). Erythrocytes 5,496,000. Hemoglobin 80 per cent.

The animal was killed by etherization on Feb. 17, 1926, for examination. Autopsy revealed marked enlargement of the lymph glands. No other changes were noticeable. The spleen, blood, and bone marrow failed to yield cultures, but growth was obtained from the emulsion of the lymph nodes.

As a result of the inoculations on the eyebrows, four tiny indurated areas appeared within 9 to 10 days but did not enlarge and within 18 days had practically disappeared. A red spot of pin-point size was noticed on the scarified area after 29 days, but it also disappeared in another week.

While the nodule employed for inoculation in this instance probably contained fewer organisms than the one removed from Monkey 4 earlier in its evolution, yet the suspension from it yielded a pure growth of *Bartonella bacilliformis* in a 1:10 dilution, and a large number

of organisms were found in sections. Loss of virulence may have resulted from the final etherization of Monkey 4.

The comparatively mild infection, accompanied by severe febrile reactions, seems to have been due to a higher resistance of this particular animal, but the chronic nature of the *Bartonella* infection is indicated even in this instance by the persistence of the microorganisms in the lymph glands.

The Reproduction of Striking Clinical Features of Oroya Fever and Verruga.

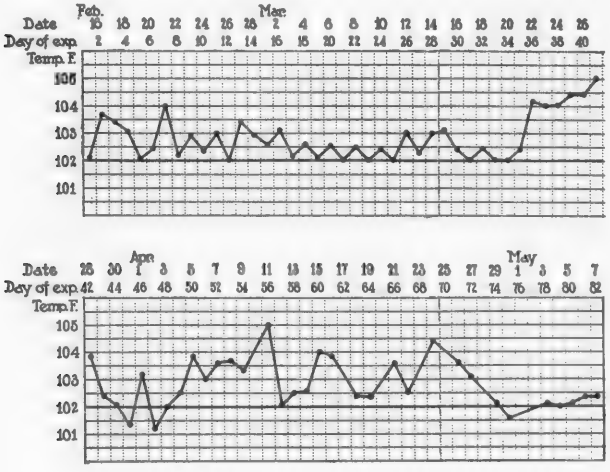
Up to this point in the work, the manifestations by monkeys of infection with *Bartonella bacilliformis* resembled human Oroya fever or verruga in essentials, *i.e.*, in the typical localization of the organism and in the characteristic skin lesions, respectively, but the severe anemia of Oroya fever and the general eruption of verruga had not as yet been reproduced in monkeys.

In Monkey 18, however, the local lesions, while not general or spontaneous, attained extraordinary size and resembled those described by previous investigators as resulting from the inoculation of suspensions of human verruga tissues into monkeys. Monkey 25 developed the spontaneous generalized eruption characteristic of verruga and also severe symptoms of Oroya fever (anemia, presence of *Bartonella bacilliformis* in considerable numbers in the erythrocytes). Although the period of fever was very brief in this animal, the manifestations of infection were similar to those of certain human cases in which Oroya fever and verruga are simultaneously present.[5]

M. rhesus 18 (Text-fig. 5) was inoculated on Feb. 15, 1926, with passage virus (suspension of nodular tissue) from Monkey 5. The shaved left eyebrow received two intradermal injections of the emulsion, and the shaved right eyebrow was scarified and smeared with the same material. The skin of the abdomen was shaved, and on the left side two intradermal injections were made of the emulsion, while on the right two scarified areas (about 3 × 3 cm. each) were spread with the same material. Induration at the sites of inoculation became evident in about 14 days and were definite after 26 days (Fig. 1). The lesions steadily increased in size, and 36 days after inoculation the largest of the nodules on the scarified areas of the right eyebrow measured 2.5 × 2 cm. and was 1 cm. high

[5] Odriozola, E., La maladie de Carrion, Paris, 1896.

(Fig. 7), while the two round subcutaneous nodules on the left abdominal wall stood out about 1 cm. and showed a reddish spot at the point of insertion of the syringe needle (Fig. 8). The lesions were of maximum size 59 days after inoculation (Fig. 2) and remained stationary for about 14 days. Retrogression was slow, but at the time of writing (99 days after inoculation) they are pale, fibrous, and considerably decreased in size, as shown in Figs. 3 and 11. No ulceration or softening of the lesions could be detected at any time. The examination of a nodule excised from the right eyebrow 26 days after inoculation revealed the characteristic granulomatous structure with proliferation of endothelial cells (Fig. 9), in the cytoplasm of which were found varying numbers of *Bartonella bacilliformis*, usually in clumps (Fig. 10).



TEXT-FIG. 5.

Bartonella bacilliformis was demonstrated in sections of the lesions and isolated in culture from emulsions of the tissue. It was recovered from the blood of the monkey on the following occasions:

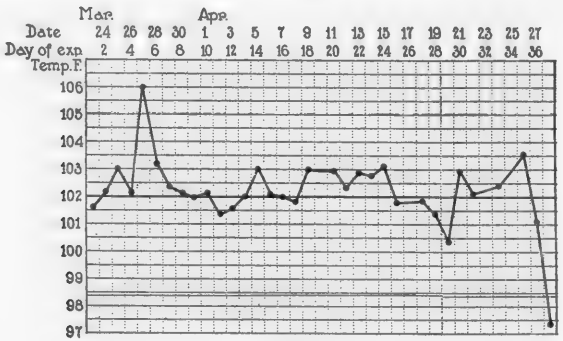
Mar. 5, 1926 (18 days after inoculation). Positive in blood diluted 1:100,000.
" 18, " (31 " " "). " " " " 1:100,000.
" 27, " (40 " " "). " " " " 1:10.

Comparison of blood counts made on the day of inoculation and 60 days later show that there was a definite decrease in the number of red cells during the course of illness:

Feb. 15, 1926. Erythrocytes 5,356,000. Hemoglobin 90 per cent.
Apr. 16, " " 4,120,000. " 60 " "

One of the most striking features of the experimental *Bartonella* lesions is the slow continuous formation of granulomatous tissue on a scarified surface of the skin, which results in an enormous accumulation of endothelial cells and subsequent capillary formation, a process apparently identical with that which takes place in the lesions of verruga in man.

M. rhesus 25 (Text-fig. 6) was inoculated intravenously on Mar. 23, 1926, with 6 cc. of a saline suspension of cultures grown on blood slants for 10 days at 25°C. At the same time the shaved right eyebrow was inoculated intradermally as well as by scarification with a suspension of nodular tissue from *M. rhesus* 18. The left eyebrow was similarly inoculated with culture. This animal had



TEXT-FIG. 6.

only 1 day of high temperature, Mar. 27, when 106°F. was recorded; otherwise the course of disease was practically afebrile.

The first sign of infection in this instance was a peculiar condition of the upper eyelids, the edges of which were irregularly thickened, and perhaps a little pinkish on Apr. 7, 15 days after inoculation. Within a week numerous miliary, cherry-red nodules appeared around the eyes on both sides, and distinct nodules became noticeable at the site of intradermal inoculation as well as on the scarified areas. The animal presented a striking aspect (Fig. 4). These lesions, and several miliary nodules scattered over the body, especially near the right inguinal surface (Fig. 5) gradually became larger within the next 5 or 6 days and partly confluent. On the posterior surface of the left leg nodules of varying size appeared within 3 weeks after inoculation (Fig. 6) and remained until death. The eyes were virtually closed 36 days after inoculation (Apr. 28, 1926), when the animal died (Fig. 12). One of the spontaneous nodules was removed for cultural

and histological examination on Apr. 15. It showed the usual endothelial proliferation (Fig. 13), and large numbers of *Bartonella bacilliformis* were present (Fig. 14).

The number of red corpuscles infected with *Bartonella bacilliformis* was larger than in any of the monkeys previously studied, cells invaded by the parasites being readily demonstrable in film preparations. Curiously enough, however, the detection of the organism was not accomplished, even by the culture method, during the 48 hours preceding death.

Mar. 27 (5 days after inoculation). Positive with blood diluted 1:1,000.
Apr. 8 (18 " " "). " " " " 1:100,000.
" 16 (26 " " "). " " " " 1:1,000,000.

The results of blood counts by Dr. Bauer were as follows:

Mar. 27, 1926 (4 days after inoculation). Erythrocytes 5,288,000. Hemoglobin 85 per cent.

Apr. 20, 1926 (28 days after inoculation). Erythrocytes 3,240,000. Hemoglobin 45 per cent.

Apr. 22, 1926 (30 days after inoculation). Erythrocytes 2,992,000. Hemoglobin 40 per cent.

Apr. 24, 1926 (32 days after inoculation). Erythrocytes 3,368,000. Hemoglobin 40 per cent.

Apr. 26, 1926 (34 days after inoculation). Erythrocytes 2,736,000. Hemoglobin 35 per cent.

Apr. 27, 1926 (35 days after inoculation). Erythrocytes 2,120,000. Hemoglobin 30 per cent.

Apr. 28, 1926 (36 days after inoculation). Erythrocytes 1,624,000. Hemoglobin 25 per cent.

Autopsy.—Very much emaciated, wax-yellow. The nodules on the eyebrows remained unopened, but the lesions on the lids had partly ulcerated, and the tissues had become somewhat necrotic. The eyes were almost closed. There was some seropurulent discharge from the ulcerated nodules, which were nearly confluent. Corneæ not affected. Spontaneous miliary nodules were found on the abdomen, thighs, and legs, the posterior surfaces of both legs, in particular, being covered with cutaneous or subcutaneous nodules of varying size (Fig. 6), some adherent to the fascia. Some nodules were hemorrhagic.

There was exudative pericarditis, due to the presence of minute Gram-negative, motile bacilli in enormous numbers, especially within the polymorphonuclear leucocytes. The heart muscles were pale and flabby, but the pleuræ did not appear to be involved. Lungs normal. Liver perhaps enlarged, and pale. Spleen somewhat enlarged and soft. Stomach empty, normal. Intestines and mesentery: General lymphatic system hypertrophied everywhere. Other organs normal. Bone marrow (femur) dark red.

The findings described suggest a terminal secondary bacterial pericarditis, which may have been the result of transfer by the animal

of infective material from the ulcerated eyelid to a needle wound caused by heart puncture. Notwithstanding this late stage invasion, the essential features of the course of illness in this animal were, I believe, the result of local and constitutional infection with *Bartonella bacilliformis*.

SUMMARY.

The experiments reported here were carried on in the main with passage strains of *Bartonella bacilliformis*, and the results indicate that the virulence of the organism has been considerably enhanced by passage through susceptible animals. While the animals of the earlier experimental series showed no anemia, some of the present group manifested a definite reduction in the number of red cells and in hemoglobin, and in one instance (*M. rhesus* 25) anemia was of the extreme type so often associated with Oroya fever in man. The anemic condition appeared to be secondary in character, however, nucleated red cells being few in number. In this animal also *Bartonella bacilliformis* was readily demonstrated in the erythrocytes by means of stained smears, though the number of cells invaded by the parasites was by no means so great as in the human infection.

In most instances of experimental *Bartonella* infection so far induced the demonstration of the parasites by ordinary routine examination of stained film preparations is possible only when the titer of the blood exceeds 1:1,000. Prolonged search of many slides has not been attempted, however. The number of microorganisms in the blood, as shown by culture tests of ascending dilutions, was in most instances highest (1:100,000 to 1:10,000,000) during the early period of the infection coincident usually with the period of highest fever, falling to a titer of 1:10 during the last half of the disease. In one of the fatally infected monkeys, however, the titer increased from 1:10 on the 4th day to 1:1,000,000 on the 24th day. The titer of the blood was equally great in Monkeys 5 and 6, although the former was inoculated locally, the other intravenously and intraperitoneally. The largest proportion of infected red cells was found in Monkey 25, while the blood titer, as shown by culture test, was highest in Monkey 7.

The febrile reaction varied in the animals of this series from a severe continuous fever of 104–105°F., lasting 2 to 3 months, in one instance,

with a remittence during the 3rd to 5th weeks, to the acute high fever (106°F.) of 1 day's duration in the fatally infected monkey, No. 25. The more usual reaction, however, is an irregular course of moderate fever with one or more periods of high temperature (105°).

Bartonella bacilliformis was constantly demonstrated, both microscopically and by culture tests, in the lymph glands of animals sacrificed 2 to 3 months after inoculation, and in two of three instances it was present also in the spleen, bone marrow, and heart blood. In the case of *M. rhesus* 6, which died 26 days after inoculation, the microorganism was obtained also in culture from the lymph glands, spleen, and heart blood taken at autopsy. In the other animal which died, a terminal bacterial infection, while not obscuring the effects of the *Bartonella* infection, made it impossible to isolate the parasite from either blood or tissues.

The skin lesions, whether of the nodular type, induced by introduction of the virus intradermally or by application to the scarified skin, or of the miliary character occurring spontaneously as a result of systemic infection, always yielded cultures of *Bartonella bacilliformis*, and stained sections of such lesions revealed the parasites in large numbers in their characteristic situation in the endothelial cells.

A chronic, systemic infection, in which the lymph glands are enlarged and *Bartonella bacilliformis* is present in the blood in high titer, may be induced by local inoculation, as shown in the case of *M. rhesus* 5.

The local lesions induced in one instance by introduction of a passage strain, both intradermally and by scarification, attained within 2 months extraordinary size, the nodules arising at adjacent sites of inoculation on the right eyebrow having coalesced into a large pedunculated mass which overhung the eye. This type of reaction had not been observed hitherto in the course of the present study but has been described by earlier investigators as a result of the inoculation of monkeys with human verruga tissues.

The striking fact brought out in the present study is the variety of responses to inoculation which animals of the same species may manifest. The clinical features of the infection may be typical of Oroya fever or may resemble those of verruga peruviana, and in *M. rhesus* 25 we have an instance of a type of infection in which the

characteristic phenomena of both conditions are simultaneously present. Whether the appearance will resemble those of the one or the other condition appears to depend on the susceptibility of the individual as well as on the virulence of the organism. Moreover, it seems probable that different degrees of resistance to the invasion of the parasite on the part of the blood cells, internal organs, or skin of a given animal may determine the predominant clinical manifestations of the infection. The factor of variation in susceptibility of different individuals or different tissues of the same individual would account for the variety of types of human *Bartonella* infection.[6]

EXPLANATION OF PLATES.

PLATE 22.

All figures natural size.

FIG. 1. *M. rhesus* 18, 26 days after inoculation. The nodule on the inner aspect of the left eyebrow was subsequently removed for examination.

FIG. 2. *M. rhesus* 18, 59 days after inoculation, when the nodules had reached their maximum development. The nodules on the right side had been induced by inoculation by scarification; there was no softening or ulceration of these lesions at any time. The nodule on the left side was induced by intradermic inoculation.

FIG. 3. *M. rhesus* 18, 99 days after inoculation, when the nodules had retrogressed considerably and the animal was decidedly paler.

FIG. 4. *M. rhesus* 25, 23 days after inoculation. The indurations which had developed at the sites of inoculation on the inner aspect of each eyebrow were almost eclipsed by the numerous spontaneous deep red nodules which had appeared about the eyes.

FIG. 5. *M. rhesus* 25, showing the spontaneous nodules which developed in the inguinal region, 29 days after inoculation.

FIG. 6. *M. rhesus* 25, showing the spontaneous nodules which had appeared on the posterior surface of the legs 29 days after inoculation.

PLATE 23.

FIG. 7. *M. rhesus* 18, 36 days after inoculation, showing the appearance of the lesions at the sites of intradermal inoculation and of inoculation by scarification. Natural size.

FIG. 8. *M. rhesus* 18, 36 days after inoculation, showing the lesions on the abdomen. The verrucous lesions on the right side were induced by scarification,

[6] Arce, J., La enfermedad de Carrion, Lima, 1920.

the two round subcutaneous nodules on the left side by intradermal and partly subcutaneous inoculation. Natural size.

FIG. 9. Section of nodule removed from eyebrow of *M. rhesus* 18, 26 days after inoculation. Giemsa's stain. × 182.

FIG. 10. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

FIG. 11. *M. rhesus* 18, 99 days after inoculation, showing the appearance of the receding lesions on the abdomen. One of the nodules at the sites of intradermal inoculation had been removed.

PLATE 24.

FIG. 12. *M. rhesus* 25, at the time of death 36 days after inoculation.

FIG. 13. Section of spontaneous nodule of *M. rhesus* 25, removed 23 days after inoculation. Giemsa stain. × 182.

FIG. 14. The same section at a magnification of 1,000 times, showing the presence of masses of *Bartonella bacilliformis*.

FIG. 1. *M. rhesus* 18,
26 days after inoculation.

FIG. 4. *M. rhesus* 25,
23 days after inoculation.

FIG. 2. *M. rhesus* 18,
59 days after inoculation.

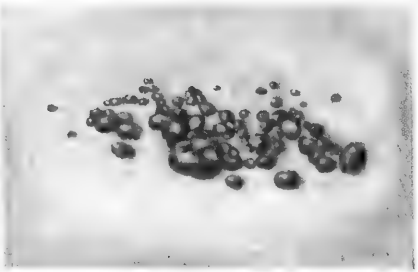


FIG. 5. *M. rhesus* 25
(groin), 29 days after inoculation.

FIG. 3. *M. rhesus* 18,
99 days after inoculation.

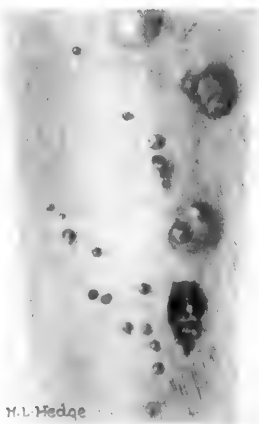


FIG. 6. *M. rhesus* 25
(leg), 29 days after inoculation.

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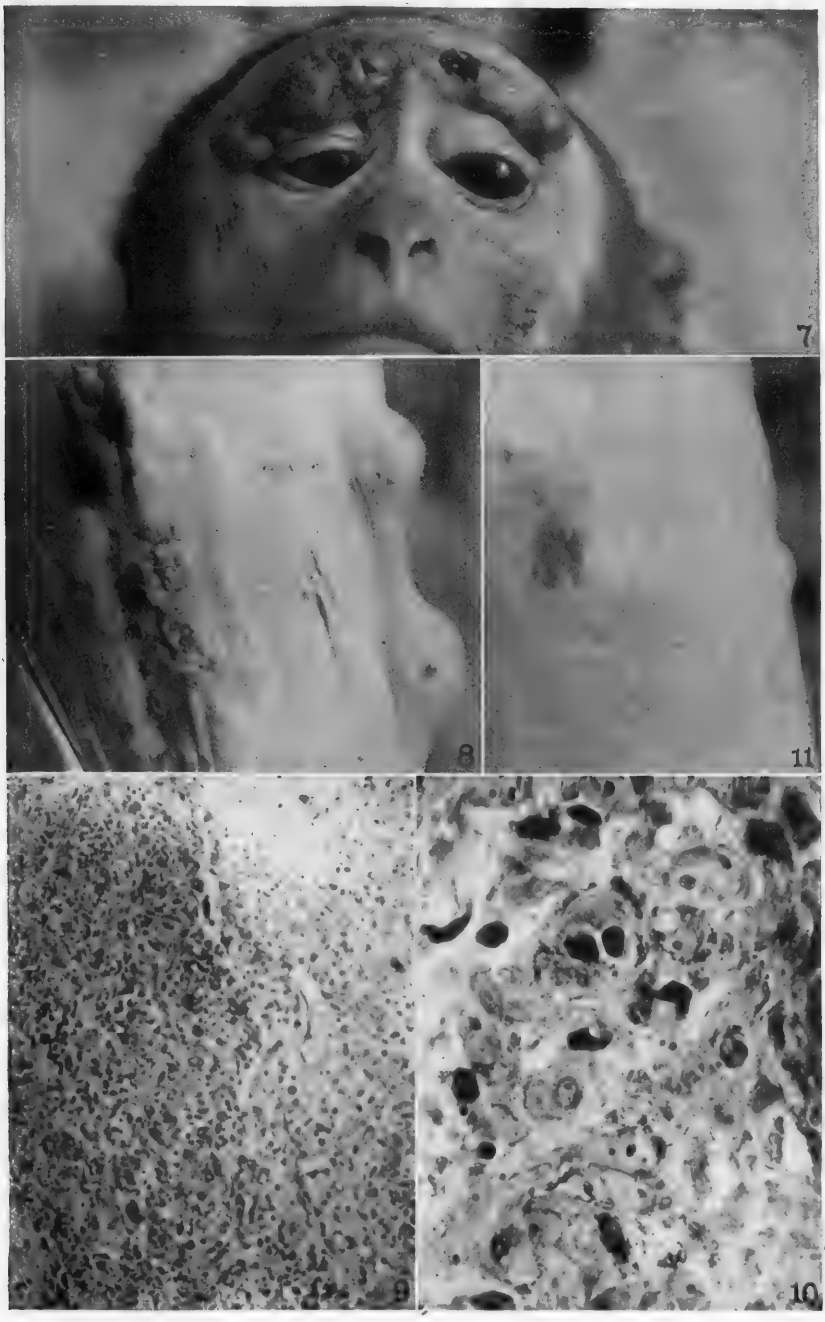
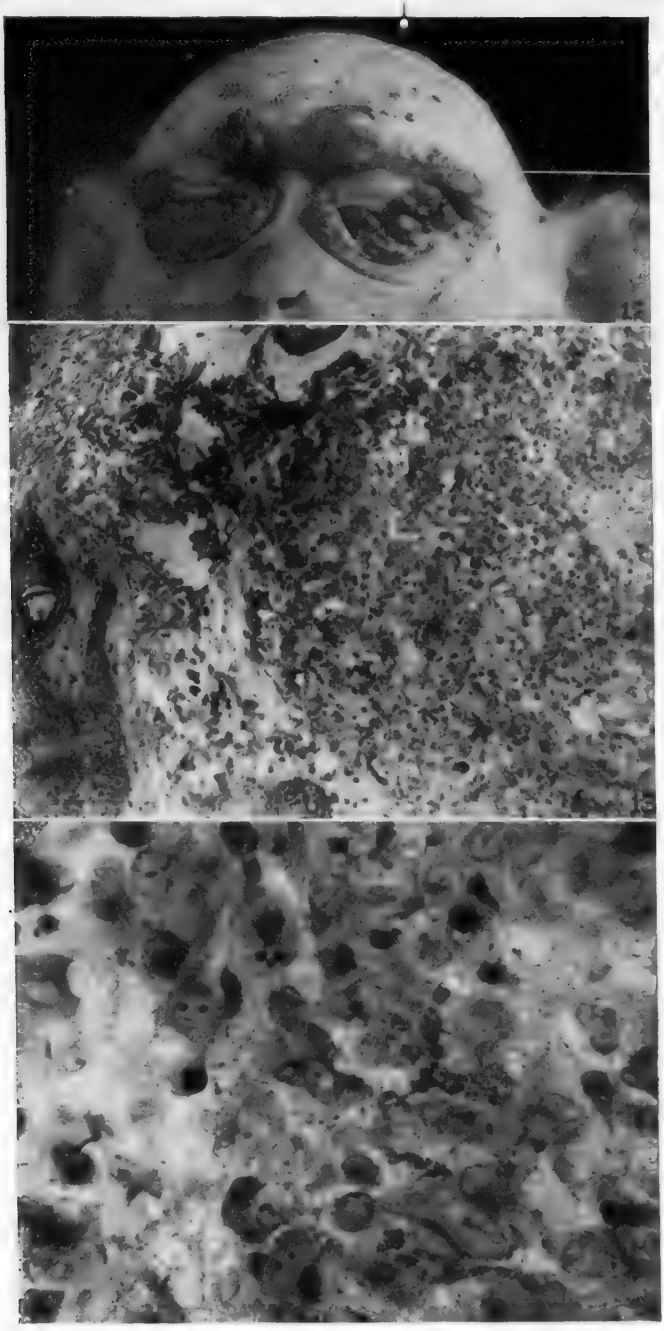


(Noguchi: Etiology of Oroya fever. III.)



(Noguchi: Etiology of Oroya fever. III.)

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ETIOLOGY OF OROYA FEVER.

IV. The Effect of Inoculation of Anthropoid Apes with Bartonella bacilliformis.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 25 to 28.

(Received for publication, June 18, 1926.)

It has been reported in previous papers that the intravenous inoculation of young *rhesus* monkeys with a strain of *Bartonella bacilliformis* isolated from a case of Oroya fever gives rise to two essential manifestations of Oroya fever, namely, protracted fever, and typical localization of the parasite within the red blood cells (Fig. 1), while the introduction of the organism intradermally induces skin lesions which are indistinguishable from those of verruga peruviana.[1] It has been shown also that after intradermal inoculation the organism may pass into the circulation and invade the red blood cells, that it may give rise to an anemic condition comparable with that of Oroya fever, except for the absence of appreciable numbers of nucleated red cells in the blood stream, and that it may induce a generalized eruption similar to that of human verruga.[2] In most instances, however, the inoculated monkeys have shown little or no anemia, and comparatively few erythrocytes are invaded by the parasite, while the verruga nodules have usually arisen only at the sites of inoculation into the skin.

Since the invasion of the red corpuscles by the bacilliform organisms is the most characteristic sign of Oroya fever, and the formation of nodules by the proliferation of endothelial cells is equally typical of verruga, the findings described indicated that the organism cultivated was probably the cause of both these conditions. Nevertheless, it

[1] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1925, xliii, 851.

[2] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

was desirable to reproduce, if possible, the complete clinical picture of Oroya fever,—the extreme anemia of pernicious type, and the invasion of large numbers of the erythrocytes by the microorganism, such as takes place in human Oroya fever (Fig. 2). The opportunity presented itself of studying the effect of the inoculation of two anthropoid apes with cultures and passage strains of *Bartonella bacilliformis*, and the experiments were undertaken in the hope that these animals, because of their close phylogenetic relationship to man,[3,4] might prove sufficiently susceptible to the infection to manifest all of the severe symptoms to which the parasite gives rise in man, either of the type of Oroya fever, or that of verruga. As the protocols show, however, they manifested only slight constitutional reaction to the inoculations. The erythrocytes were invaded by the organisms to a small extent only, and there was little or no anemia.[5] The skin lesions, while of the characteristic verruga type, remained localized at or near the sites of introduction of the microorganisms; there was no spontaneous generalized skin eruption such as is observed in human verruga, and in rare instances in *rhesus* monkeys.

A young female chimpanzee (*Pan leucoprymnus*), said to be about 4 years old, was inoculated on Jan. 29, 1926, with the suspension of a nodule excised* from the eyebrow of *M. rhesus* 3[1] on the same day, and also with cultures, grown on leptospira medium, representing the second generation from the original human blood and the first generation from the blood of *M. rhesus* 1.[1] The suspension was inoculated intradermally into the right eyebrow and by scarification on the right side of the abdomen, while on the left eyebrow and left side of the abdomen a mixture of the suspension with cultures was introduced part intradermally and part subcutaneously. The quantities injected were 0.2 to 0.3 cc. 5 cc. of the mixture were injected subcutaneously into the right lower portion of the abdomen, above the inguinal region, the site of inoculation being carefully massaged after the injection.

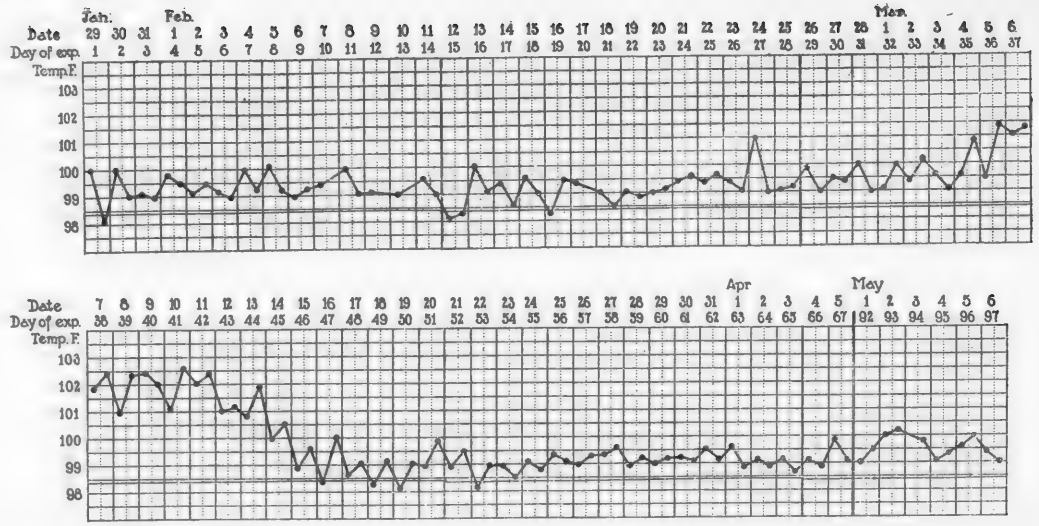
The normal temperature of the chimpanzee, as shown by daily records covering a period of several weeks, varied between 98.6°F. and 99.6°; occasionally the afternoon (4 p.m.) temperature was as high as 100°. Blood counts made before inoculation on Jan. 29 gave the following results: Erythrocytes 5,520,000, leuco-

[3] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

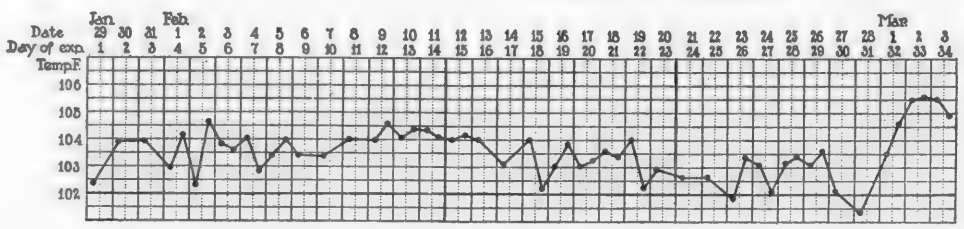
[4] Landsteiner, K., and Miller, C. P., *J. Exp. Med.*, 1925, xlii, 853.

[5] The hematological studies were by Dr. J. H. Bauer.

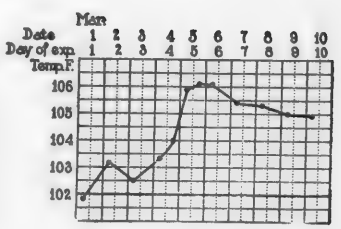
* All operations were performed under ether anesthesia.



TEXT-FIG. 1. Chimpanzee.



TEXT-FIG. 2. *M. rhesus* 14.



TEXT-FIG. 3. *M. rhesus* 21.

cytes 7,810, hemoglobin 80 per cent.[6] The inoculations were made between 12 noon and 1 p.m.

Jan. 30. Animal appeared as usual; appetite good. Slight irritation at the sites of scarification. *Jan. 31.* No change, except that the lines of scarification were slightly raised. *Feb. 3.* Some swelling at the sites of intradermal injection, but no reddening. *Feb. 4.* Animal appeared perfectly well. Erythrocytes 5,680,000, leucocytes 8,190, hemoglobin 85 per cent.

Feb. 8. The abdominal wall showed definite edema, but the animal ate well and appeared normal. Erythrocytes 5,335,000, leucocytes 11,900, hemoglobin 85 per cent. The blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10, but routine examination of film preparations failed to demonstrate cells containing the organism. *Feb. 9.* Distinct induration at the sites of intradermal inoculation on eyebrows and abdomen, but no reddening. *Feb. 12.* Abdominal nodule at site of intradermal and subcutaneous injection increased in size (3 × 5 mm.); overlying skin edematous. There was a small red spot at the most prominent portion of the nodule. Animal still in excellent condition. *Feb. 15.* Reddening of abdominal nodule had spread peripherally; a tumor of irregular contour and uneven surface was felt under the tense, somewhat edematous skin. The tumor was firm, and the skin was partly adherent to it; there was no softening or fluctuation on palpation. Scarified areas on abdomen showed erythematous dots, but there was no definite change in the appearance of the scarified areas on the eyebrows. The nodules induced by the intradermal injections measured 4 × 4 mm. The induration was more subcutaneous than intradermal, and at the points of needle insertion the skin was pinkish.

Feb. 19. Abdominal nodule 1.5 × 2 cm. in size, deep seated, irregular to palpation, and partly adherent to the skin, which appeared somewhat yellowish. Abdomen less edematous. Nodules on eyebrows slightly increased in size; overlying skin normal in color. *Feb. 22.* Abdominal nodule measured 5 × 6 cm. and stood out prominently above the abdominal wall. It seemed to consist of several lobulous nodules of varying size. The skin showed a definitely yellowish tint on one side. The lymph glands in the axillary and inguinal regions and at the flexor side of both elbow joints were markedly swollen.

Feb. 23. The abdominal nodule was harder and perhaps somewhat larger (Fig. 13). The lines of scarification on the right abdominal wall were bright red, considerably raised, and indurated (Fig. 9). Erythrocytes 6,234,000, leucocytes 12,000, hemoglobin 90 per cent. The blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000, and the organism was demonstrated in small numbers in the red cells. *Feb. 25.* The intradermal nodules on the eyebrows (Fig. 8) were red at the apices. The other lesions had increased in size and intensity.

Mar. 1. The animal had shown no constitutional reaction and appeared to be perfectly well. Erythrocytes 5,120,000, hemoglobin 85 per cent. Cultures of

[6] All the hemoglobin estimates were made with Sahli's hemoglobinometer.

undiluted blood positive. Routine examination of blood films failed to reveal blood corpuscles containing *Bartonella bacilliformis*. A portion of the abdominal nodule (Fig. 3) was excised for examination. It showed characteristic proliferation of endothelial cells (Fig. 14), and *Bartonella bacilliformis* was demonstrable in the cytoplasm of the cells (Figs. 5, 15). Cultivation of a suspension of the nodular tissue yielded growth of the organism. A portion of the skin lesion on the scarified area of the abdomen (Fig. 9) was also excised. It was similar in structure to the abdominal nodule (Figs. 10, 11) and yielded a culture of *Bartonella bacilliformis*.

For more than four weeks the blood count, temperature, appetite, and general health and disposition of the animal had remained apparently normal. There were so few microorganisms in the blood on Mar. 1 that undiluted citrated blood in amounts less than 0.2 cc. failed to yield growth, and demonstration of red corpuscles containing *Bartonella bacilliformis* was not possible by the ordinary routine examination of one or two film preparations. The effect of the inoculation had become mainly local, although the lymph glands were still much enlarged.

At this point it seemed desirable to determine the effect of reinoculating the animal with a virulent passage strain from a *rhesus* monkey. *M. rhesus* 14 (Text-fig. 2), which had been inoculated in the same way as the chimpanzee and at the same time, had developed large nodules on eyebrow and abdomen by Feb. 23 (Figs. 16, 20), and the one at the former site had been excised on Feb. 27 for examination. The abdominal nodule (Figs. 21, 22) was excised on Mar. 1, and suspensions of both nodules and of a piece of skin from one of the scarified areas on the abdomen (Figs. 18, 19) were mixed with cultures of *Bartonella bacilliformis* and inoculated intravenously into the chimpanzee. The same material was injected into *M. rhesus* 21 as a test of its virulence.

An accident intervened at this time. *M. rhesus* 14, which had served as the control for the first inoculation of the chimpanzee and was showing far more active and rapidly progressing local and constitutional reactions, had been used in some experiments to determine whether or not *Bartonella bacilliformis* could be transmitted by insects. Thirty ticks (*Dermacentor andersoni*, believed to be free from spotted fever) were allowed to feed on the animal from Feb. 25 to Feb. 27.

All became attached. The animal had been having irregular fever (104°F. or slightly above) for the past 4 weeks, and the unintentional infection with spotted fever by tick feeding escaped attention for a time. When the animal died on Mar. 4, however, it became evident that death was due to spotted fever, and that *M. rhesus* 21 (Text-fig. 3) and the chimpanzee had received on Mar. 1 Rocky Mountain spotted fever virus as well as *Bartonella bacilliformis*. *M. rhesus* 21 died of typical spotted fever within 10 days (4 days incubation, 6 days of high fever, 106°F. during the first 2 days). Notwithstanding the presence of the spotted fever infection, the blood of Monkeys 14 and 21 both yielded cultures of *Bartonella bacilliformis*, that of *M. rhesus* 21 in a dilution of 1:100,000. Injections into guinea pigs of the blood and of suspensions of the nodular tissue from Monkey 14 induced typical fatal spotted fever.

Spotted Fever in the Chimpanzee.—As the chart shows (Text-fig. 1), the spotted fever infection (Mar. 1 to 15) gave rise in the chimpanzee to a very different temperature curve from that of the pure infection with *Bartonella bacilliformis* (Jan. 29 to Mar. 1). After an incubation period of 5 days the temperature rose to 101°F., then gradually to 102.5°F., then fell to a slightly subnormal level, and remained there for several days. The fever was relatively mild, as compared with that usually observed in spotted fever infection in man, *Macacus rhesus* monkeys, and guinea pigs; in these animals spotted fever gives rise to a temperature of 105–106° lasting 7 to 10 days. The chimpanzee had become inactive on the 5th day, was disinclined to move about, depressed, and indifferent to food. This condition continued for 5 days (until Mar. 9). By this time the accident had become known through the death of *M. rhesus* 14, and 20 cc. of pooled spotted fever antiserum from hyperimmunized rabbits were given intravenously to the chimpanzee and also to the control *rhesus* (No. 21). In the *rhesus* the injection had no effect; the animal died the following day. In the chimpanzee the character of the fever before and after the administration of the serum appeared to be the same, and the recognition of curative effect is difficult unless the short duration of the fever is assumed to have been due to the effect of the serum. In *rhesus* monkeys and guinea pigs, however, the serum has not been known to influence the course of fever unless given during the incubation period.[7] At all events, the chimpanzee recovered very rapidly and probably without aid of the antiserum.

During the period of spotted fever infection, when the animal was very ill, all the nodules, including the reddened skin lesions, became paler and smaller,

[7] Noguchi, H., *J. Exp. Med.*, 1923, xxxvii, 383.

and the face of the animal was cyanotic, though the color had returned within a week after the time of injection of the serum.

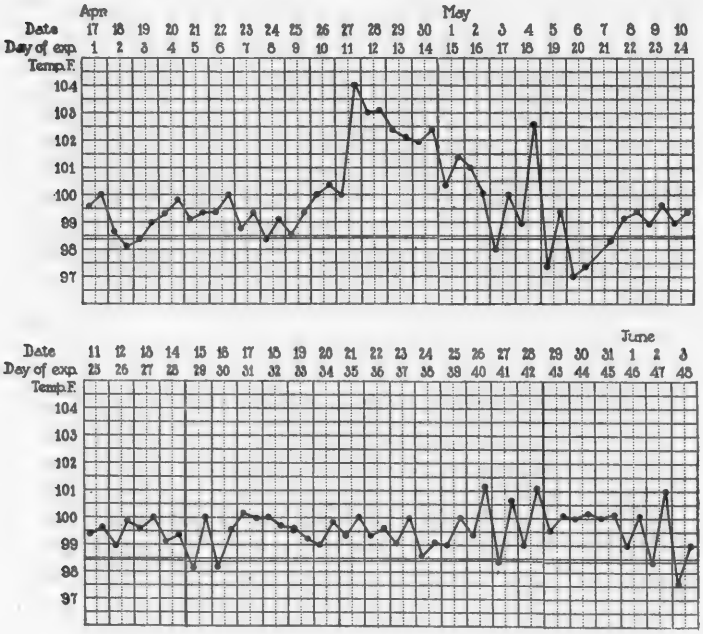
Mar. 1, 1 p.m. Intravenous injection of 1.5 cc. of a mixture of suspension of nodular tissue of *M. rhesus* 14 (removed Feb. 27 and Mar. 1) and cultures of *Bartonella bacilliformis*. *Mar. 5.* The animal ate very little and was inactive. The nodules on the eyebrows were still large (Fig. 12). *Mar. 6.* Temperature 101°F. *Mar. 7.* Temperature 102° a.m., 102.5° p.m. *Mar. 8.* Temperature 101° a.m., 102.5° p.m. Animal inactive, apathetic, appetite poor. The abdominal wounds, where the lesions had been removed, had partly opened, and the surrounding tissues were edematous. *Mar. 9.* Temperature 102.5° a.m., 102° p.m. The animal was very ill. The nodules were pale and cyanotic. Erythrocytes 4,856,000, hemoglobin 80 per cent. 20 cc. spotted fever antiserum given intravenously at 12 noon.

Mar. 10. Temperature 101° a.m., 102.5° p.m. The color had returned to the nodules, and the animal was somewhat more active. Appetite improved. *Mar. 11.* Temperature 102° a.m., 102.4° p.m. The animal was more active, and the color of the face and nodules more nearly normal. *Mar. 12.* Temperature 101° a.m., 101.2° p.m. Eyelids edematous. Erythrocytes 4,288,000, hemoglobin 65 per cent. Blood yielded growth of *Bartonella bacilliformis* in dilution of 1:100. *Mar. 13.* Temperature 101° a.m., 101.8° p.m. The monkey had removed some of the stitches from the wounds caused by excision of the lesions; the wounds were healing satisfactorily, however. *Mar. 14.* Temperature 100.5° a.m., 100.5° p.m. *Mar. 15.* Temperature 99° a.m., 99.5° p.m. Nodules smaller and paler, not cyanotic. Erythrocytes 3,488,000, hemoglobin 60 per cent. Blood culture negative. One of the inguinal glands on the left side was removed for examination. The suspension yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10. *Mar. 17.* Temperature 98.4° a.m., 99° p.m. The animal was very active. Erythrocytes 4,344,000, hemoglobin 65 per cent. *Mar. 20.* Temperature 99.5° a.m., 100° p.m. The remaining nodules had diminished considerably in size and the overlying skin was of normal color. *Mar. 25 to 31.* Temperature normal. The skin lesions had healed and showed slight brownish pigmentation. The site of the abdominal nodule was practically flat, and the induration had disappeared. The nodules on the eyebrows were reddish but small (3 × 4 mm.).

Apr. 2. The nodules on the eyebrows were much smaller. The lymph nodes had also decreased in size considerably. *Apr. 6.* Blood culture negative. Erythrocytes 4,456,000, hemoglobin 75 per cent. *May 1.* The nodules had practically disappeared. Neither the blood nor the lymph yielded cultures of *Bartonella bacilliformis*. *May 3.* Erythrocytes 5,800,000, hemoglobin 85 per cent. The animal had completely recovered.

The Behavior of Bartonella bacilliformis in the Ourang-Utan.

The response of the ourang-utan to the inoculation of *Bartonella bacilliformis* was similar to that of the chimpanzee, but in this instance there was a moderate febrile reaction (Text-fig. 4) which began 10 days after inoculation and lasted about a week. On the day before the onset of fever *Bartonella bacilliformis* was demonstrable



TEXT-FIG. 4. Ourang-utan.

by culture in a 1:100 dilution of the blood, but a week later the titer of the blood was only 1:10. The local lesions, while characteristic of those induced by the organism, did not approach in severity those usually observed in *rhesus* monkeys. The largest nodules and the slowest to pass away were two which arose subcutaneously at the sites of attempted intravenous inoculation into the basilic vein of the left arm (Fig. 24), and which remained prominent for 2 months.

They had disappeared, however, by Aug. 15 (4 months after inoculation).

The ourang-utan (*Pongo pygmæus*), a young female (probably 2 to 3 years old), was inoculated Apr. 17, 1926. The right eyebrow was scarified and smeared with a piece of nodule just excised from *M. rhesus* 23 and was intradermally inoculated, on the inner aspect, with 0.2 to 0.3 cc. of a suspension of the same tissue. 0.2 cc. of the suspension was injected intradermally into the shaved skin of the right upper abdominal wall, and about 1 cc. was smeared over a scarified area on the right lower side of the abdomen. The left side of the abdomen was inoculated similarly at two sites with a mixture of cultures derived from the blood of *M. rhesus* 7 and *M. rhesus* 18. The mixture included cultures grown on leptospira medium for 10 days and blood agar cultures 6 days old which contained a large number of motile organisms.

The animal received intravenously a mixture containing 6 cc. of the cultures, 1 cc. of the suspension of nodular tissue of *M. rhesus* 23, and 3 cc. of citrated blood from each of two monkeys, *M. rhesus* 11 and *M. rhesus* 24. Two attempts to introduce the syringe needle into the basilic vein of the left arm having failed, the material was inoculated into the corresponding vein of the right arm. A small amount of material from the syringe had, however, been unintentionally introduced subcutaneously into the left arm, and two large subcutaneous nodules later developed at the sites of the attempted intravenous injections.

A blood count made on Apr. 2, 1926, had shown 5,940,000 erythrocytes per c.mm., 12,200 leucocytes, and 85 per cent hemoglobin. The normal temperature of the animal had been taken daily at 11 a.m. and at 4 p.m. for a period of several weeks. It varied from 98.6°F. to 99.6° and was rarely as high as 100° in the afternoon.

Blood taken on Apr. 26, 9 days after inoculation, yielded growth of *Bartonella bacilliformis* in a dilution of 1:100, and a few cells containing the organism were found in film preparations. The erythrocytes at this time numbered 5,392,000, and the hemoglobin was 70 per cent. For the 10 days following inoculation the temperature remained within normal limits, but on the afternoon of Apr. 27 it rose to 104°, and on Apr. 28 it was 103° both morning and afternoon.

Apr. 29. Temperature 102.4° a.m., 102.2° p.m. Appetite poor. Lymph glands enlarged. Slight induration at site of needle puncture on right arm. Erythrocytes 4,880,000, hemoglobin 75 per cent. *Apr. 30.* Temperature 102° a.m., 102.4° p.m. Animal quiet and apathetic. *May 1.* Temperature 100.4° a.m., 101.4° p.m. *May 2.* Temperature 101° a.m., 100° p.m. *May 3.* Temperature 98° a.m., 100° p.m. Diarrhea. Blood yielded growth of *Bartonella bacilliformis* in dilution of 1:10. Erythrocytes 4,808,000, hemoglobin 80 per cent. Slight induration at sites of intradermal injection on each eyebrow.

May 4. Temperature 99° a.m., 102.6° p.m. *May 5.* Temperature 97.4° a.m., 99.4° p.m. *May 6.* Temperature 97° a.m., 97.4° p.m. Distinct nodules

at sites of inoculation on eyebrows and areas of induration at the sites of the attempted intravenous injections on the left arm.

From this time on the temperature varied within normal limits. On May 11 the erythrocytes numbered 5,632,000, and the hemoglobin was 80 per cent. Blood taken on May 17 yielded cultures of *Bartonella bacilliformis*. The induration at the site of intradermal inoculation on the right eyebrow had disappeared by May 13, but the adjacent lines of scarification had become distinctly raised and reddish, and there was a distinct nodule, 5 × 6 mm., at the site of intradermal inoculation on the left eyebrow. The area of scarification on the lower right side of the abdomen also showed activity, being markedly raised and reddish. *May 20.* Distinct nodules on left side of abdomen at sites of inoculation of cultures. One of these was excised, and also a piece of skin from the scarified area on the lower right abdomen. Both showed the characteristic structure of lesions induced on the skin by *Bartonella bacilliformis*, and the organisms were demonstrated in sections of the tissue (Figs. 6, 25, and 26).

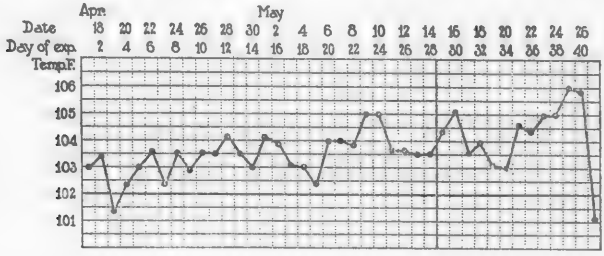
May 23. One of the nodules on the left arm measured 2.5 × 3 cm., the other 5 × 5 mm. (Fig. 24). The nodule on the left eyebrow was about 8 × 9 mm., and there were numerous small reddish papules on the scarified area of the right eyebrow (Fig. 23). All the lesions continued to increase in size until about June 1, when the photographs shown in Figs. 23 and 24 were taken. *June 2.* Blood culture negative for *Bartonella bacilliformis*. Erythrocytes 5,512,000, hemoglobin 80 per cent, leucocytes 11,600. Polymorphonuclears 33.3 per cent, small lymphocytes 45.25 per cent, large lymphocytes 3.8 per cent, large mononuclears 11.0 per cent, transitionals 1.0 per cent, eosinophils 0.7 per cent, mast cells 4.75 per cent.

The monkey scratched the nodule on the left eyebrow until on June 8 she had succeeded in opening it and on June 10 had removed nearly half of it. The remaining portion was excised on June 10 for examination and culture (Fig. 4). The papules on the right eyebrow had become dry and scaly at this time, and the abdominal lesions had practically disappeared.

In the *rhesus* monkey, inoculated with the same material as the ourang-utan to serve as a control, the local reactions were less severe than those often observed in this species, but there was a tendency to generalization of the skin lesions, and the systemic reaction was very severe. Within 7 days after inoculation the sites of intradermal injection on the eyebrows were definitely indurated, and there was a slight spontaneous eruption on the abdomen. The temperature rose on the 11th day after inoculation, and there was continuous high temperature (104–106°F.) after the 20th day (Text-fig. 5). The hemoglobin at the time of death, 40 days after inoculation, was 15 per cent, and the erythrocytes numbered 1,176,000.

M. rhesus 37 was injected intravenously (saphenous vein) on Apr. 17, 1926, with the same mixture of cultures and nodular suspension which the ourang-utan had received. The right eyebrow was inoculated intradermally and by scarification with the suspension of the nodule of *M. rhesus* 23, the left in the same way with the mixture used for intravenous injection. The shaved skin of the lower right side of the abdomen was inoculated by scarification with the nodule suspension of *M. rhesus* 23, the lower left side with a suspension of nodular tissue of *M. rhesus* 18. The latter suspension was also inoculated by scarification on two sites on the upper abdomen.

Apr. 23. Slight induration at the sites of intradermal inoculation on the eyebrows. *Apr. 24.* Indurations on the eyebrows definite; slight spontaneous eruption on abdomen. *Apr. 28.* Temperature 104.2°F. Distinct nodule at site of intravenous injection on the right leg and red papules at all four sites of scarification on abdomen. *Apr. 29.* Blood yielded growth of *Bartonella bacilliformis* in dilution of 1:10. Erythrocytes 5,180,000, hemoglobin 70 per cent.



TEXT-FIG. 5. *M. rhesus* 37.

May 1. Temperature 104.2°. *May 3.* Nodule on left eyebrow 2 × 3 mm. *May 7.* Lines of scarification on right eyebrow slightly raised. Nodules on left eyebrow and on leg increased in size. *May 12.* Erythrocytes 5,536,000, hemoglobin 75 per cent. *May 19.* Blood culture negative.

From May 19 to May 24 this animal was used for the feeding of nineteen ticks (*Dermacentor variabilis*). On May 20 there was a spontaneous papular eruption on the abdomen. The nodules on the eyebrows had not increased in size, but the one on the right leg was 4 × 5 mm. On May 26, when the temperature was 105.8° the animal was used for the feeding of ten bedbugs, which were removed the following morning. *May 27.* Temperature 101°; animal very ill, cyanotic. *May 28.* Died.

Autopsy.—Profuse hemorrhage on the abdominal wall and in the omentum but no free blood in the peritoneal cavity. Peritoneal fluid yellowish, slightly turbid, contained a few red cells. Intestines smooth and glistening, no evidence of peritonitis. Lungs whitish gray, extremely anemic, but otherwise normal.

No fluid in the pericardium, no evidence of pericarditis. Heart muscle heavily congested and very hemorrhagic. Liver very pale and soft but showed no gross pathological changes. Spleen normal in size but very dark in color and much harder than normal. Kidneys normal in appearance. There were a few dark bluish enlarged lymph nodes in the mesenterium and one in the large intestine, but otherwise the intestinal tract appeared normal. Axillary and inguinal lymph glands very slightly enlarged. One small nodule on the left eyebrow and another larger one on the back of the left leg. Both on section showed the presence of *Bartonella bacilliformis*.

Erythrocytes 1,176,000, leucocytes 3,200, hemoglobin 15 per cent. Polymorphonuclear leucocytes 50.7 per cent, small lymphocytes 31.1 per cent, large lymphocytes 3.2 per cent, large mononuclears 11.8 per cent, transitionals 1.9 per cent, eosinophils 0.8 per cent, mast cells (basophils) 1.25 per cent, megaloblasts 1.0 per cent.

The red cells stained very irregularly, a large number of them remaining practically unstained, while about 25 per cent showed marked polychromatophilia. There were a large number of microcytes and a few macrocytes, mostly of irregular shape. No nucleated red cells were found. A few red cells contained *Bartonella bacilliformis*. There were a few threads of fibrin in the smears but no platelets.

The majority of the polymorphonuclear leucocytes had become so changed that the cytoplasm had practically disappeared. The nucleus was still intact. Most of the lymphocytes were extremely small, about half the size of the red cells.

SUMMARY.

The inoculation of a chimpanzee with cultures and a passage strain of *Bartonella bacilliformis* induced local reactions which, while definite and characteristic, progressed less rapidly and were much less striking than those in the control *rhesus* monkey. *Bartonella bacilliformis* was demonstrated in the blood corpuscles with difficulty, and the fever was slight compared with the high and persistent fever of the *rhesus* monkey. In both the swelling of the lymph glands was an early symptom and constantly present. Definite anemia developed in the chimpanzee only after accidental infection with Rocky Mountain spotted fever and may have been due to either one or both infections, though it disappeared when the blood had become negative by culture for *Bartonella bacilliformis* and the local lesions had disappeared. Incidentally, the chimpanzee was found in this one instance to be less susceptible to the spotted fever than *Macacus rhesus* and guinea pigs.

In the ourang-utan, also, *Bartonella bacilliformis* induced a mild systemic and local infection. A rise of temperature occurred 10 days after inoculation, and fever continued for a week, though it was decidedly less severe than that in the control *rhesus*. The lesions induced by scarification were less definite than those which arose at the sites of intradermal inoculation. *Bartonella bacilliformis* was recovered from the blood on the 9th and on the 16th days after inoculation and from nodules excised on the 33rd and 53rd days. A few erythrocytes containing the organism were demonstrated in stained smears, but prolonged search was required to find them.

The symptoms and lesions observed in the chimpanzee and ourang-utan as a result of infection with *Bartonella bacilliformis* are far milder than those of *rhesus* monkeys and show less resemblance to human Oroya fever or verruga.

EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. *Bartonella bacilliformis* in the erythrocytes of *Macacus rhesus*. Selected cells from film preparations of the blood of *M. rhesus* 25 and *M. rhesus* 30. Giemsa's stain. × 1,750.

FIG. 2. *Bartonella bacilliformis* in the erythrocytes of Case S. A. 15, from which the organism was isolated. Giemsa's stain. × 1,750.

FIG. 3. The nodule excised from the abdomen of the chimpanzee 31 days after inoculation. Natural size and color.

FIG. 4. The nodule excised from the eyebrow of the ourang-utan 53 days after inoculation. Natural size and color.

FIG. 5. *Bartonella bacilliformis* in a section of the chimpanzee nodule shown in Fig. 3. Giemsa's stain. × 1,750.

FIG. 6. *Bartonella bacilliformis* in a section of a nodule of the ourang-utan, excised 33 days after inoculation. Giemsa's stain. × 1,750.

FIG. 7. *Bartonella bacilliformis* in a section of a spontaneous nodule of *M. rhesus* 25 excised 23 days after inoculation. For comparison. Giemsa's stain. × 1,750.

PLATE 26.

FIG. 8. Appearance of the nodules on the eyebrows of the chimpanzee 27 days after inoculation. 1/2 natural size.

FIG. 9. The skin lesion on the right side of the abdomen of the chimpanzee as it appeared 25 days after inoculation.

FIG. 10. Section of the lesion shown in Fig. 9, which was excised 32 days after inoculation. Giemsa's stain. × 182.

FIG. 11. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

FIG. 12. The appearance of the nodules on the eyebrow of the chimpanzee 31 days after inoculation.

FIG. 13. The abdominal nodule of the chimpanzee as it appeared 25 days after inoculation.

FIG. 14. Section of the nodule shown in Fig. 13, which was excised 32 days after inoculation. Giemsa's stain. × 182.

FIG. 15. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

PLATE 27.

FIG. 16. Appearance of the nodules on the eyebrows of *M. rhesus* 14, 23 days after inoculation. Natural size.

FIG. 17. The skin lesions on the abdomen of *M. rhesus* 14, 23 days after inoculation. Natural size.

FIG. 18. Section of one of the skin lesions shown in Fig. 17, excised 23 days after inoculation. Giemsa's stain. × 182.

FIG. 19. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

FIG. 20. The abdominal nodule of *M. rhesus* 14, 23 days after inoculation. Natural size.

FIG. 21. Section of the nodule shown in Fig. 20, which was excised 28 days after inoculation. Giemsa's stain. × 182.

FIG. 22. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.

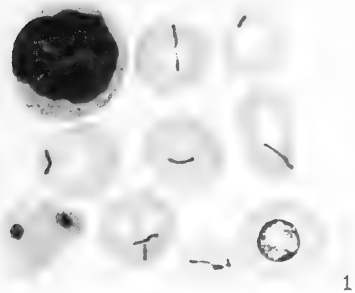
PLATE 28.

FIG. 23. Appearance of the skin lesions on the eyebrows of the ourang-utan 43 days after inoculation. 2/5 natural size.

FIG. 24. The large subcutaneous nodules which arose on the left arm of the ourang-utan at the site of unsuccessful attempts to enter the basilic vein at the time of inoculation. Photograph taken 43 days after inoculation. 2/5 natural size.

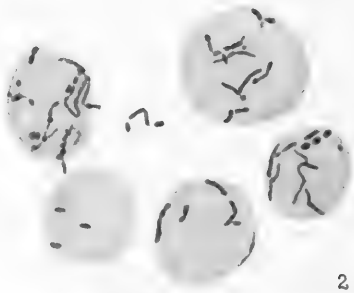
FIG. 25. Section of the abdominal nodule of the ourang-utan, excised 40 days after inoculation. Giemsa's stain. × 182.

FIG. 26. The same section at a magnification of 1,000 times, showing the presence of *Bartonella bacilliformis*.



1

Blood of *M. rhesus*.



2

Human blood.

3

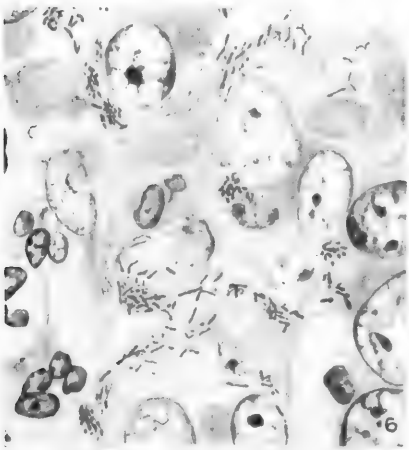
Abdominal nodule, chimpanzee.

4

Eyebrow nodule, ourang-utan.

5

Bartonella in chimpanzee nodule.



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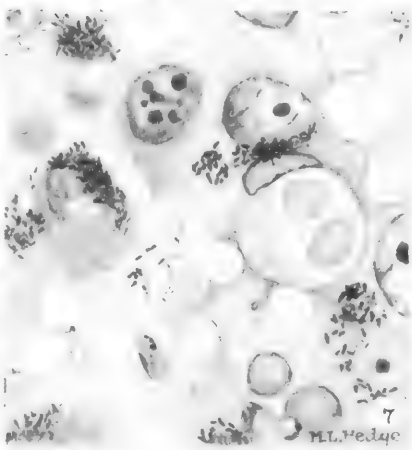
Bartonella in ourang-utan nodule.



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Bartonella in nodule of *M. rhesus* 25.

(Noguchi: Etiology of Oroya fever. IV.)

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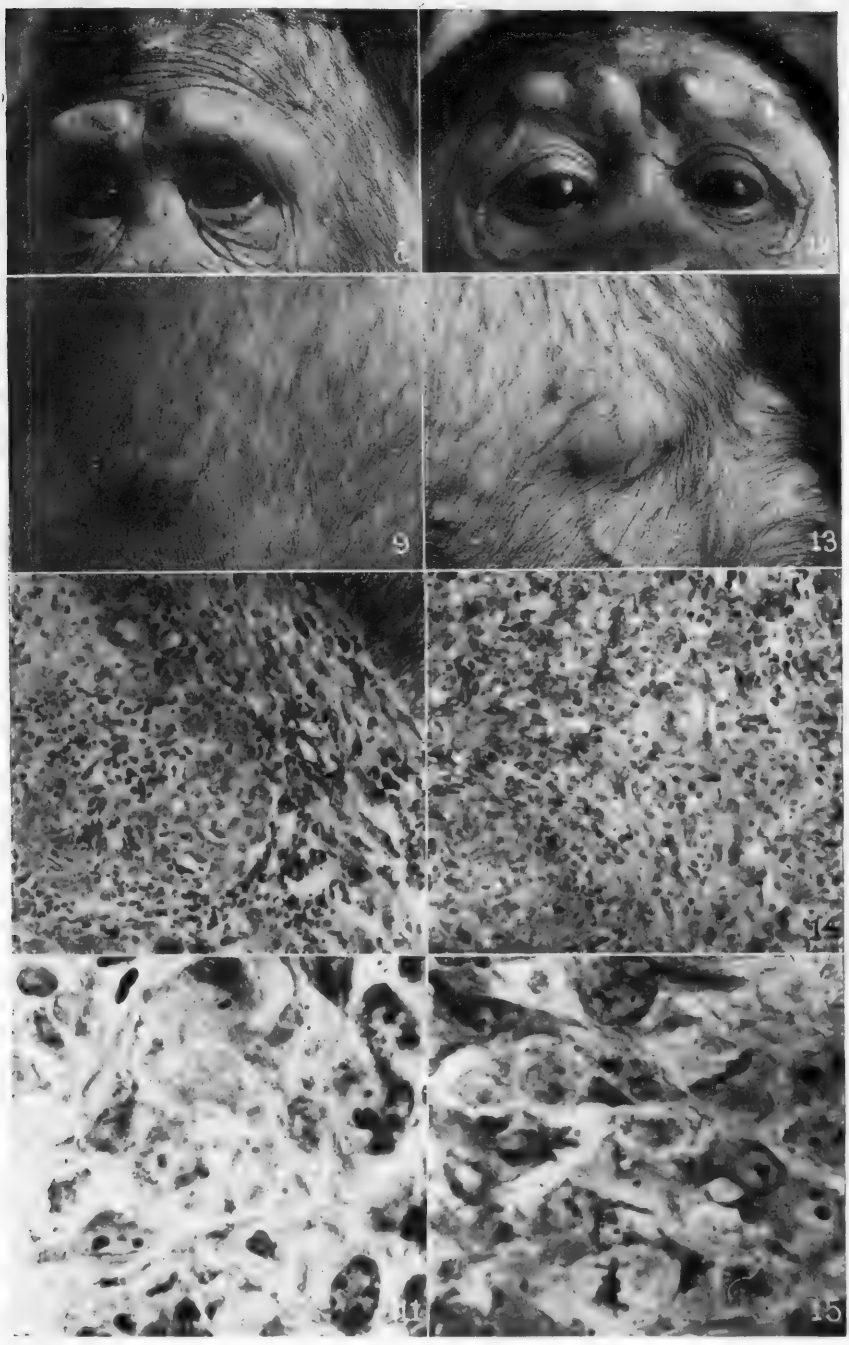

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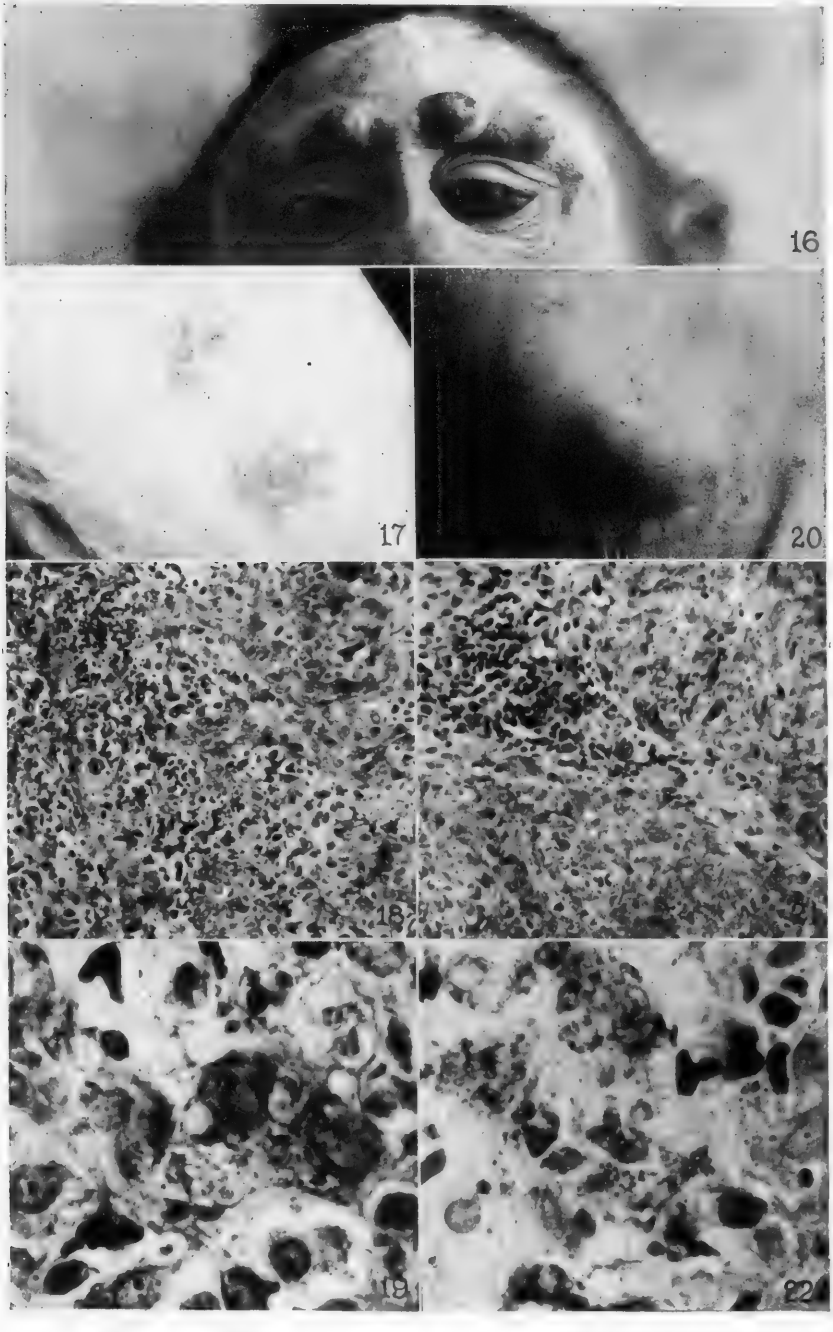

(Noguchi: Etiology of Oroya fever. IV.)

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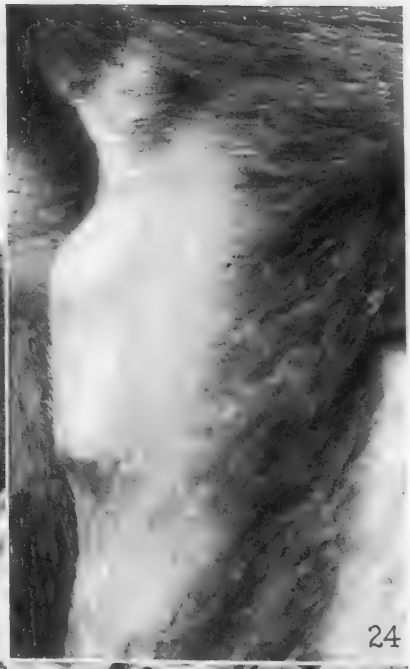
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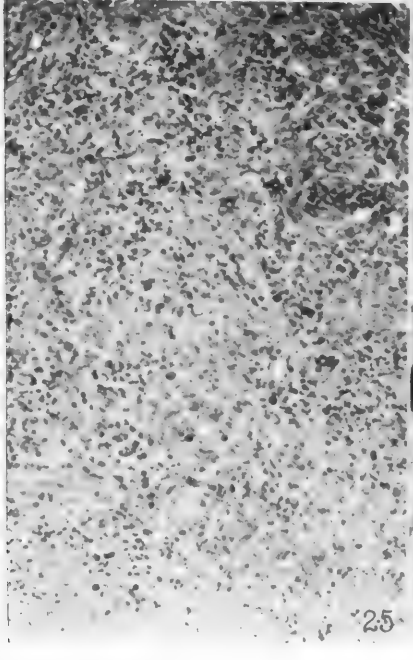
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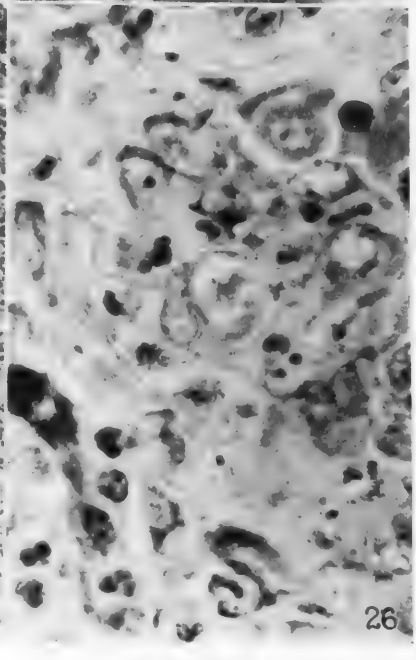
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(Noguchi: Etiology of Oroya fever. IV)

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ETIOLOGY OF OROYA FEVER.

V. The Experimental Transmission of Bartonella bacilliformis by Ticks (Dermacentor andersoni).

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The geographical distribution of Oroya fever, which occurs within certain limited areas of Peru, has suggested to many students of disease that infection is carried by some biting insect or arthropod, and Castellani and Chalmers[1] note particularly the analogous localization of the tick-borne disease, Rocky Mountain spotted fever. Entomological investigation in relation to Oroya fever has not so far implicated any particular insect, with the possible exception of *Phlebotomus verrucarum* Townsend,[2] and experimental work has been hampered by the lack of animals susceptible to infection with *Bartonella bacilliformis* and methods of cultivating the organism. These obstacles have recently been overcome,[3,4] however, and the presence of *Bartonella bacilliformis* in insects can now be definitely determined both by cultural methods and by infection experiments. There is considerable evidence,[3–5] moreover, that verruga peruviana is also caused by *Bartonella bacilliformis*, as had long been suspected because of the similar geographical distribution of the two conditions and their frequent association in the same individuals.

It was not possible at the moment to undertake an investigation of insects from the infected districts, but an indirect method of approach to the problem suggested itself, that of determining whether

[1] Castellani, A., and Chalmers, A. J., A manual of tropical medicine, London, 3rd edition, 1919.

[2] Townsend, C. H. T., *J. Am. Med. Assn.*, 1913, lxi, 1717.

[3] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

[4] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

[5] Noguchi, H., and Hercelles, O., *Science*, 1926, lxiv, 121.

some well known carrier of infectious agents—such, for example, as the tick which transmits Rocky Mountain spotted fever,[6] tularemia,[7] and a filterable virus pathogenic for guinea pigs[8]—could also act as a vector of *Bartonella bacilliformis* under experimental conditions. Success in transmitting the parasite under such circumstances, while not solving the problem of the actual vector, would nevertheless furnish a starting point for the investigation of the question.

For the specimens of ticks (*Dermacentor andersoni*) used in these experiments, I am indebted to Dr. R. R. Parker, of the United States Public Health Service, Hamilton, Montana. The ticks were first allowed to feed on normal guinea pigs to determine whether or not they were free from the spotted fever virus. If the guinea pigs escaped infection, the ticks were then placed on the shaved abdominal skin of monkeys (*Macacus rhesus*) in various stages of infection with *Bartonella bacilliformis* and allowed to feed for periods varying from 24 hours to 6 days. Tests of infectiveness were made by feeding the ticks on normal *rhesus* monkeys either immediately following their removal from the infected animal or after an interval of 12 to 14 days.

Two series of experiments were made. In the first, the periods of tick feeding were relatively short—24 hours to 4 days, and several days were allowed to elapse between the removal of the ticks from the infected animals and the test feedings. Infection not being obtained under these conditions, in the second series the period of infective feeding was lengthened to 5 days, the ticks were transferred to normal animals immediately after their removal from the infected ones, and 6 days were allowed for the test feeding. Under these circumstances the ticks proved infective.

Experiment 1 (*Negative*).

Feb. 1, 1926. Thirty ticks were placed on three infected monkeys (Nos. 7, 12, and 14), ten on each animal, and allowed to remain attached for 1 to 4 days.

[6] Ricketts, H. T., *J. Infect. Dis.*, 1907, iv, 141.
[7] Francis, E., *J. Am. Med. Assn.*, 1925, lxxxiv, 1243.
[8] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 1.

M. rhesus 7[4] had been inoculated intravenously and intradermally on Dec. 21, 1925 (41 days previously), with blood and suspensions of nodular tissue from Monkeys 2, 3, and 4. *Bartonella bacilliformis* was present in the blood 4 days after injection and on several occasions during the following month. The local lesions were well developed by Jan. 19 and were excised* on that date. From Jan. 27 to Feb. 1 fever had been continuous, fluctuating between 104° and 105°F. The ticks were left attached for 24 hours (Feb. 1 to 2). Fever continued until Feb. 9. The blood had yielded cultures of *Bartonella bacilliformis* previously in dilutions as high as 1:10,000,000, but it developed subsequently that the titer had fallen to 1:10 at the time of the tick feeding.

M. rhesus 12 had been inoculated intravenously and intradermally on Jan. 19, 1926, with first generation cultures, derived from the blood of Monkeys 4, 6, and 7, and grown for 14 days on leptospira medium. The blood was positive by culture on Jan. 25 and on Feb. 3 (6 and 15 days after inoculation). The intradermal injections gave rise to small nodules, which, however, never progressed to typical lesions but gradually receded within a month to mere traces of induration. The temperature was continuously high (104°) from Jan. 30 to Feb. 5. The ticks were allowed to feed 72 hours (Feb. 1 to 4).

M. rhesus 14[4] had been inoculated on Jan. 29, 1926, with a suspension of nodular tissue from *M. rhesus* 3 and with cultures of *Bartonella bacilliformis* derived from human and monkey blood, the material having been introduced intradermally, subcutaneously, and by scarification. The temperature rose after 24 hours, and the animal remained febrile (104–104.6°F.) for 4 days. The ticks were allowed to feed on the animal Feb. 1 and 2. Blood taken on Feb. 8 yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10 only. The local and general symptoms progressed steadily until on Feb. 23 the blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000, the nodules on the eyebrows were very large and protruding, and those on the abdomen measured 1.5 × 3 cm. It is evident that the ticks were placed on the animal too early in the course of the infection.

Feb. 16, 1926. Three ticks of each lot of ten were placed on the shaved abdominal skin of *M. rhesus* 19, and two of each lot were dissected and suspensions of the viscera inoculated intradermally into the shaved skin of the eyebrows and abdomen of *M. rhesus* 20. The suspensions were tested also by culture. No infection could be demonstrated in the ticks, and the cultures were negative for *Bartonella bacilliformis*.

M. rhesus 19. Nine ticks, of which three had been fed on *M. rhesus* 7, three on *M. rhesus* 12, and three on *M. rhesus* 14, were placed on the shaved skin of the

* All operations were performed under ether anesthesia.

abdomen on Feb. 16, 1925, and allowed to remain 4 days. All became attached. The animal showed no signs of infection—no enlargement of the lymph nodes, fever, or skin lesions. Because of prolapse of the rectum, the animal was killed by etherization on Mar. 6, 18 days from the time of tick feeding. Nothing abnormal was found at autopsy, and cultures of heart blood, bone marrow, spleen, lymph nodes, liver, kidneys, testis, and lungs were negative for *Bartonella bacilliformis*.

M. rhesus 20. Inoculated Feb. 16, 1926, intradermally into the shaved skin of eyebrows and abdomen with the mixed suspensions of the viscera of ticks fed on Monkeys 7, 12, and 14. There were small indurations at the sites of intradermal inoculation on the eyebrows after 10 days, but they disappeared within 3 weeks. The abdominal skin showed no reaction to the injections. The temperature rose to 104° or higher (104.6°) on several occasions (Feb. 19, 21, and 24, Mar. 7 and 11). No swelling of the lymph nodes was noted during 42 days of observation.

Experiment 2 (Positive).

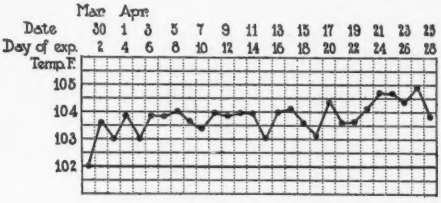
Four ticks were allowed to feed on each of two infected monkeys (*M. rhesus* 18 and *M. rhesus* 23) for 5 days, Mar. 24 to 29, 1926.

M. rhesus 18[4] had been inoculated on Feb. 15, 1926, intradermally and by scarification on the shaved eyebrow with a suspension of nodular tissue from *M. rhesus* 5. On Mar. 5 and 18 the blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100,000. The nodules on the eyebrows became extremely large. During the time of tick feeding (Mar. 24 to 29) the temperature fluctuated daily between 104° and 105°. On Mar. 27 the blood was positive in a dilution of 1:10.

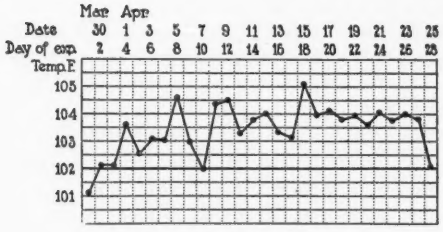
M. rhesus 23 had been inoculated Mar. 9, 1926, intravenously and by scarification with fourth generation cultures derived from *M. rhesus* 7, and intradermally with a suspension of the nodule excised from *M. rhesus* 18 on that date. The local inoculations induced in time very striking nodules and indurations, and blood taken on Mar. 22 yielded growth of *Bartonella bacilliformis* in a dilution of 1:100,000. The temperature was 104°F. on Mar. 24, when the ticks were placed on the abdominal skin. All were moderately engorged when removed on Mar. 29. The course of infection in this animal subsequently became very severe both locally and systemically. There was a prolonged period of fever, and 2 weeks later spontaneous miliary eruptions appeared on the face. The animal was sacrificed May 1, 42 days after inoculation.

On removal from the infected animals, each lot of ticks was immediately placed on a normal *rhesus* monkey and allowed to feed for a period of 6 days. After the feeding the ticks were dissected, and mixed suspensions of the viscera were inoculated into another normal

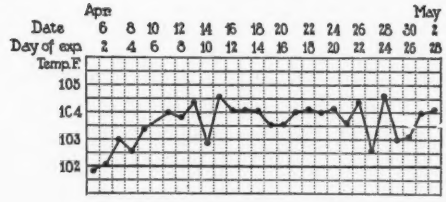
monkey. Definite, though mild, infection was induced in all the animals, and *Bartonella bacilliformis* was recovered in culture from the blood and lymph nodes. The suspensions of the tick viscera also yielded cultures of the organism.



TEXT-FIG. 1. *M. rhesus* 26.



TEXT-FIG. 2. *M. rhesus* 27.



TEXT-FIG. 3. *M. rhesus* 28.

M. rhesus 26. Mar. 29, 1926. Four ticks which had been allowed to feed for 5 days on *M. rhesus* 18 were placed on the shaved skin of the abdomen and left there for 6 full days. On removal, two of the four were found dead, the other two were well engorged. The animal began to show irregular mild fever (Text-fig. 1) 11 days from the time the feeding began, and the lymph glands in

the inguinal and axillary regions on both sides were somewhat swollen. On Apr. 14 one of the inguinal lymph nodes was excised for examination. A suspension of the tissue yielded cultures of *Bartonella bacilliformis*. The temperature was 104–105°F. from Apr. 21 to 25, and blood taken on Apr. 24 yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10. The enlargement of the lymph glands has steadily progressed up to the time of writing (May 30, 1925.) No skin lesions have developed.

M. rhesus 27. Mar. 29, 1926. Four ticks which had been allowed to feed for 5 days on *M. rhesus* 23 were placed on the shaved skin of the abdomen and allowed to feed for 6 full days. All the ticks became attached and were moderately engorged at the time of removal. The reaction in this animal was much the same as that in the foregoing monkey. Cultures made with a suspension of one of the inguinal lymph nodes excised on Apr. 14 and with blood withdrawn on Apr. 24 were positive in dilutions of 1:10. The animal has shown irregular febrile reactions (Text-fig. 2), but no skin lesions have developed up to the time of writing.

M. rhesus 28 (Text-fig. 3). Apr. 5, 1926, inoculated intradermally on the left eyebrow with a suspension in 1 cc. saline of two ticks fed on *M. rhesus* 26 and on the right eyebrow with a suspension in 2 cc. saline of four ticks fed on *M. rhesus* 27. Six-tenths of a mixture of the two suspensions was injected intravenously. The course of events in this animal was very similar to that in Monkeys 26 and 27. Small nodules appeared at the sites of intradermal injection on the eyebrows about 8 days after inoculation, but they did not progress further. The lymph node removed from the inguinal region on Apr. 14 and blood withdrawn on Apr. 24 both yielded cultures of *Bartonella bacilliformis*. A few papular eruptions had appeared on May 19. The skin became yellowish in color about a month after inoculation and has remained so up to the time of writing.

SUMMARY.

Experiments are reported in which *Bartonella bacilliformis* was transmitted from infected to normal *rhesus* monkeys by the bite of the tick, *Dermacentor andersoni*. A long period of feeding, both on the infected animal and on the normal animal subjected to infection, was required in order to secure positive results. The infection transmitted by the ticks was mild, but definite, as shown by the recovery of *Bartonella bacilliformis* from the lymph nodes and blood.